(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
23 June 2005 (23.06.2005)

PCT

(10) International Publication Number
WO 2005/056759 A2

(51) International Patent Classification[7]: C12N

(21) International Application Number: PCT/US2004/040694

(22) International Filing Date: 3 December 2004 (03.12.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/527,167	4 December 2003 (04.12.2003)	US
60/581,613	21 June 2004 (21.06.2004)	US
60/601,665	13 August 2004 (13.08.2004)	US
60/619,483	16 October 2004 (16.10.2004)	US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO,

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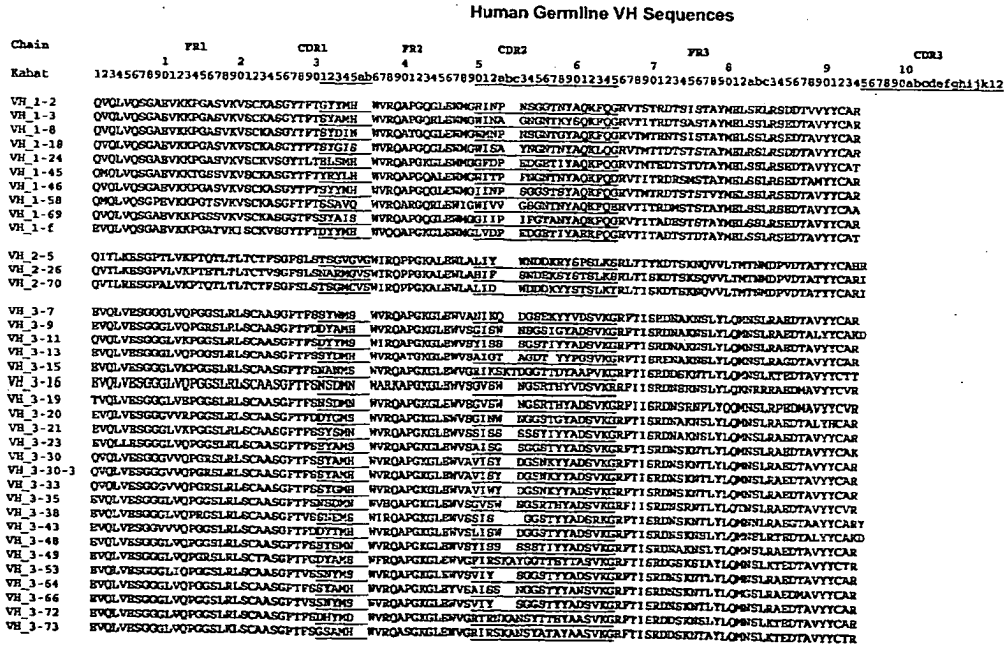
(54) Title: METHODS OF GENERATING VARIANT PROTEINS WITH INCREASED HOST STRING CONTENT AND COMPOSITIONS THEREOF



(57) Abstract: The present invention relates to novel methods for generating variant proteins with increased host string content, and

SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHODS OF GENERATING VARIANT PROTEINS WITH INCREASED HOST STRING CONTENT AND COMPOSITIONS THEREOF

[01] This application claims the benefit of under 35 U.S.C. § 119(e) to USSNs 60/527,167, filed December 4, 2003; 60/581,613, filed June 21, 2004; 60/601,665, filed August 13, 2004; and, 60/619,483, filed October 14, 2004; all of which are expressly incorporated by reference in their entirety.

FIELD OF THE INVENTION

[02] The present invention relates to novel methods for generating variant proteins with increased host string content, and proteins that are engineered using these methods.

BACKGROUND OF THE INVENTION

[03] Many proteins that have the potential to be useful human therapeutics have a xenogeneic origin. The use of xenogeneic proteins for therapeutic purposes may be advantageous for a variety of reasons, including, for example, the established success of hybridoma technology for raising antibodies in rodents, and the possibility of higher efficacy with a xenogeneic protein than with a human counterpart. Although xenogeneic proteins are a rich source of potential therapeutic molecules, they remain a relatively untapped one. One reason for this is that nonhuman proteins are often immunogenic when administered to humans, thereby greatly reducing their therapeutic utility. Additionally, even engineered proteins of human origin may become immunogenic due to changes in the protein sequence.

[04] Immunogenicity is the result of a complex series of responses to a substance that is perceived as foreign, and may include production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, hypersensitivity responses, and anaphylaxis. Several factors can contribute to protein immunogenicity, including but not limited to protein sequence, route and frequency of administration, and patient population. Immunogenicity may limit the efficacy and safety of a protein therapeutic in multiple ways. Efficacy can be reduced directly by the formation of neutralizing antibodies. Efficacy may also be reduced indirectly, as binding to either neutralizing or non-neutralizing antibodies typically leads to rapid clearance from serum. Severe side effects and even death may occur when an immune reaction is raised. One special class of side effects results when neutralizing antibodies cross-react with an endogenous protein and block its function.

[05] Because of the clinical success of monoclonal antibodies, immunogenicity reduction

of these proteins has been an intense area of investigation. Antibodies are a unique system for the development of immunogenicity reduction methods because of the large number of highly conserved antibody sequences and the wealth of high-resolution structural information. A number of strategies for reducing antibody immunogenicity have been developed. The central aim of all of these approaches has been the reduction of nonhuman, and correspondingly immunogenic content, while maintaining affinity for the antigen.

[06] The dominant method in use for antibody immunogenicity reduction, referred to as "humanization", relies principally on the grafting of "donor" (typically mouse or rat) complementarity determining regions (CDRs) onto "acceptor" (human) variable light chain (VL) and variable heavy chain (VH) frameworks (FRs) (Tsurushita & Vasquez, 2004, Humanization of Monoclonal Antibodies, Molecular Biology of B Cells, 533-545, Elsevier Science (USA)). This strategy is referred to as "CDR grafting" (Winter US 5225539). "Backmutation" of selected acceptor framework residues to the corresponding donor residues is often required to regain affinity that is lost in the initial grafted construct (US 5530101; US 5585089; US 5693761; US 5693762; US 6180370; US 5859205; US 5821337; US 6054297; US 6407213). Despite the significant clinical application of antibodies engineered using these methods, these methods remain nonrobust with regard to their ability to reduce immunogenicity. A number of humanized antibodies have elicited substantial immune reaction in clinical studies, with incidences of immune response as high as 63 % of patients (Ritter et *al.*, 2001, *Cancer Research* 61: 6851–6859).

[07] The incomplete capacity of current humanization methods for immunogenicity reduction are due to significant limitations imposed by the donor-acceptor approach. Historically, the use of a single donor has been part of methods aimed at engineering a single xenogeneic antibody to be suitable as a human biotherapeutic. However, the use of a single acceptor is not required. On the contrary, the use of an acceptor antibody, and the use of global homology to select it, place substantial restrictions on the immunogenicity reduction process. A principal problem is that the use of overall sequence similarity between nonhuman and human sequences as a metric for human immunogenicity is fundamentally flawed. This means of measuring the degree of humanness does not accurately account for the underlying molecular mechanisms of immune response. The immune system does not recognize antigens on the basis of global sequence similarity to human proteins. Rather, immune cells, including antigen presenting cells (APCs), T cells, and B cells, recognize linear or conformational motifs comprising only a handful of residues. A key step in antigen recognition is the formation of peptide-MHC-T cell receptor complexes. APCs express MHC molecules that recognize short (approximately nine residue) linear peptide sequences,

referred to as MHC agretopes. T cells express T cell receptors that recognize T cell epitopes in the context of peptide-MHC complexes. T cells that recognize MHC agretopes that are present in human proteins typically undergo apoptosis or become anergic, while T cells that recognize foreign agretopes bound to MHC molecules may participate in an immune response. Thus the relevant quantity for the immunogenicity of a protein is not its global sequence similarity to a human sequence, but rather its sequence content of individual human epitopes.

[08] The donor-acceptor model and the use of global sequence homology that it imposes fails in practice. Because CDRs are treated as inviolable, structural incompatibilities are introduced at the CDR-FR boundaries. Grafting of foreign donor CDRs onto a human acceptor framework creates a substantial number of nonhuman epitopes in each variable chain, including not only the epitopes in the foreign CDRs, but also the large number of epitopes at the FR-CDR boundaries. This FR-CDR incompatibility is evident when one backs away from global homology and looks at more local sequence homologies. CDR grafting generally maximizes the donor-acceptor homology of the frameworks at the expense of the CDRs (Clark, 2000, *Immunology Today* 21: 397-402). Ironically this frequently results in lower global homology to human antibodies. In reality, the "cut and paste" approach to imparting the functional determinants of a nonhuman antibody onto the framework of a human one is unnecessary, as careful analysis of the antigen binding determinants of antibodies shows that, in fact, the majority of CDR residues are not involved in binding antigen (MacCallum *et al.*, 1996, *J. Mol. Biol.* 262: 732-745). FR-CDR incompatibility causes not only immunological problems at the sequence level, but also causes conformational problems at the structural level. As a result, humanization methods based on CDR grafting often result in antigen affinity losses of 10-100 –fold, necessitating backmutation to donor residues within the framework. This process of backmutation is a hallmark of essentially all current humanization efforts, and because it introduces yet additional nonhuman epitopes, highlights the inefficiency of these methods.

[09] Methods that take an immune epitope approach to reducing antibody immunogenicity have been explored (US 5712120; US 2003/0153043). Central to these methods is the determination of sequences within a xenogeneic antibody that are in fact immunogenic epitopes. Different methods for determination of immunogenicity both theoretical and experimental have been described and include determination of potential for amphipathic helix formation, binding to MHC, reactivity in a T-cell activation assay. A distinguishing feature between these strategies and the present invention is that the present invention makes no presumption as to the immunogenicity of specific epitopes. Rather, the primary

goal is to maximize the content of human linear sequence strings in the xenogeneic antibody as determined by comparison to an alignment of human sequences. The relevant sequence dataset comprises strings that are nonimmunogenic for all relevant reasons, including lack of interaction with MHC, lack of interaction with T cell receptor, lack of proper processing necessary for presentation, and tolerance.

[10] It is noted that the methods described in US 5712120 and US 2003/0153043 suffer additionally in that they fail to address a significant concern for local level sequence engineering, namely the requirement for maintaining protein structure, stability, solubility, and function. Thus, although the sequence string approach to immunogenicity reduction is more accurate than CDR grafting, it will be optimal when coupled with protein design methodology that takes into account both local sequence content and conformational compatibility at the local and global structural level. In addition to providing scoring functions for assessing host string content, the present invention also describes scoring functions that evaluate other relevant properties of a protein that may be employed for the simultaneous immunogenicity reduction and structural and functional optimization of proteins.

[11] In summary, the donor-acceptor model imposes significant restrictions on the immunogenicity reduction process. With regard to sequence, global sequence homology is an inappropriate metric for immunogenicity. With regard to structure, backmutations are needed to repair conformational incompatibilities, thereby creating or reintroducing nonhuman epitopes. The present invention describes a novel method for antibody immunogenicity reduction that steps outside of the donor-acceptor model, and thus the sequence and structural restrictions it imposes. The central strategy of the described method is that it maximizes the content of human linear sequence strings. In this way immunogenicity is addressed at the local sequence level, typically by utilizing the local sequence information contained in an alignment of human sequences. This strategy not only provides a more accurate measure of the immunogenicity, it enables substitutions to be designed in a forward rather than backward manner to repair problems introduced by the graft. In effect, by addressing immunogenicity at the local sequence string level, the optimal balance between binding determinants and **humanness** can be designed.

[12] The present invention describes a novel method for reducing the immunogenicity of proteins that leverages the nonimmunogenic information contained in natural human sequences to score protein sequences for immunogenic content at the sequence string level. Furthermore, the described method capitalizes on recent advances in computational sequence and structure-based protein engineering methods to quantitatively and

systematically determine the optimal balance between human sequence content and protein functionality. Because of the wealth of human sequence information available for the immunoglobulin protein family, application to human antibodies is emphasized. Applications to other proteins are also possible.

SUMMARY OF THE INVENTION

[13] The invention disclosed herein provides a novel method for reducing the immunogenicity of a protein, wherein the method maximizes the content of sequence strings. In a preferred embodiment, the method of the present invention maximizes the content of human sequence strings.

[14] It is an object of the present invention to provide scoring functions that may be used to evaluate the human sequence string content of a protein. In a preferred embodiment, the scoring function compares the similarity of strings in a protein sequence to the strings that compose a set of natural protein sequences. In another preferred embodiment, the set of sequences is an aligned set of germline sequences. In additional preferred embodiments, the set of sequences contains mature sequences. In the most preferred embodiments, the sequences are human sequences.

[15] It is an object of the present invention to provide scoring functions that may be used to evaluate the structural and/or functional fitness of a protein.

[16] It is an object of the present invention to provide protein variants of a parent protein that are engineered using the methods described herein. In a preferred embodiment, the parent protein is an immunoglobulin.

[17] It is an object of the present invention to provide experimental methods for screening and testing the protein variants of the present invention.

[18] The present invention provides isolated nucleic acids encoding the protein variants described herein. The present invention provides vectors comprising the nucleic acids, optionally, operably linked to control sequences. The present invention provides host cells containing the vectors, and methods for producing and optionally recovering the protein variants.

[19] The present invention provides compositions comprising the protein variants described herein, and a physiologically or pharmaceutically acceptable carrier or diluent.

[20] The present invention provides novel antibodies and Fc fusions that comprise the protein variants disclosed herein. The novel antibodies and Fc fusions may find use in a

therapeutic product.

[21] The present invention provides therapeutic treatment and diagnostic uses for the protein variants disclosed herein.

BRIEF DESCRIPTION OF THE DRAWINGS

[22] Figure 1. Human germ line sequences and diversity. The sequences that are known to encode the human VH chains (Figure 1a), human VL kappa chains (Figure 1b), and VH and VL kappa J chains (Figure 1c) are shown. The VL lambda germline sequences are not provided. The germline sequences are numbered according to the numbering scheme of Kabat (Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest,* 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda). The regions of the variable region are indicated above the numbering in Figures 1a and 1b, and these include framework regions 1 through 3 and the CDRs 1 through 3. Positions that make up the Kabat CDRs are underlined. The germline chains are grouped into 7 subfamilies for V_H and and 6 subfamilies for V_L, as is known in the art, and these subfamilies are grouped together and separated by a blank line. The sequences of the five germlines that make up the IgG light kappa J chains (IGKJ1 – IGKJ5), and the six germlines that make up the IgG heavy J chains (IGHJ1 – IGHJ5) are shown in Figure 1c. The kappa and lambda light J chains combine with the VLκ and VLλ germlines respectively to form the light chain variable region, and the heavy J chains combine with the VH germlines and heavy diversity (D) germlines (not shown) to form the heavy chain variable region. The V_H CDR3 is not part of the V_H germ line, and is encoded by the D and J genes.

[23] Figures 2. The quantities described by equations 1, 2, and 3 are illustrated. In Figure 2a, IDstring (Equation 1) is illustrated for the string beginning at position i = 15, comparing a region of the murine antibody m4D5 VH sequence (VH_m4D5) as parent sequence s with the homologous region from the VH human germline sequence (VH_1-2) as human sequence h. Only 30 residues from each sequence are shown, and the residues that compose the relevant string are bolded. In Figure 2b, IDmax (Equation 2) is illustrated for the parent sequence s string that begins at position i = 15 (shown in bold) and the homologous regions from an aligned set of 7 VH human germline sequences. In Figure 2c, HSC(s) (Equation 3) is illustrated for all strings (i=1 to i=22) in the parent sequence s and the homologous regions from an aligned set of 7 VH human germline sequences.

[24] Figure 3. Sequence, host string content, and structure of WT AC10 VL. Figure 3a shows the sequence of the WT AC10 VL. Figure 3b shows the identity of each residue in WT AC10 VL as compared to the corresponding residue in each sequence of the human

VLκ germline. The black horizontal lines delineate the 7 different subfamilies as presented in Figure 1, and the black vertical lines delineate the different framework and CDR regions of the domain (in the order FR1-CDR1-FR2-CDR2-FR3-CDR3-FR4). A grey square indicates that the germline sequence has the same amino acid identity to the residue at the corresponding position in the WT AC10 VL sequence. A white square indicates that the two sequences differ at that position. Figure 3c shows the continuous 8- and 9-mer strings between WT AC10 VL and each sequence of the human VLκ germline. The black horizontal and vertical lines are as described in Figure 3b. A grey square indicates that the germline sequence comprises an 9-mer string centered on that position that is an 8 out of 9 or 9 out of 9 identical match to the corresponding string (centered on the corresponding residue) in the WT AC10 VL sequence. Figure 3d shows the structure of the modeled WT AC10 variable region. The light chain is shown as grey ribbon, the heavy chain is shown as black ribbon, and the CDR residues are indicated as black lines.

[25] Figure 4. Sequence, host string content, and structure of WT AC10 VH. The figure is as described in the figure legend for Figure 3, except that here the light chain is shown as black ribbon and the heavy chain is shown as grey ribbon.

[26] Figure 5. Sequence, host string content, and structure of CDR grafted AC10 VL. CDR grafted AC10 VL was derived from the CDRs of WT AC10 and the frameworks of the human germline sequence vlk_4-1. Differences between CDR grafted AC10 VL and WT AC10 VL are shown as bolded residues in the sequence in Figure 5a, and as black ball and sticks in Figure 5d.

[27] Figure 6. Sequence, host string content, and structure of CDR grafted AC10 VH. CDR grafted AC10 VH was derived from the CDRs of WT AC10 and the frameworks of the human germline sequence vh_1-3 and substitutions Q108L and A113S (Kabat numbering) in FR4.

[28] Figure 7. AC10 VL and VH variants with optimized HSC. AC10 VL and VH variants with optimized HSC. The nonredundant set of output sequences from the calculations described in Example 1 are shown. For each iteration (Iter) the following are provided: the Structural Consensus; Structural Precedence; Human String Content (HSC); Human String Similarity (HSS); N_9max; the Framework Region Homogeneity (FRH); and, the number of mutations from WT (Muts). The output sequences were clustered based on their mutational distance from the other sequences in the set. These clusters are delineated by the horizontal black lines. The "Cluster" column provides this mutational distance quantitatively. Differences between the parent WT AC10 sequence are shown in grey. Positions are

numbered according to the Kabat numbering scheme, provided at the top. The light grey regions bracketed by the black horizontal lines indicate residues in or proximal to the Kabat defined CDRs that were masked in the calculation. Sequence differences from WT C225 VL are shown in dark grey.

[29] Figure 8. Sequence, host string content, and structure of L1 AC10 VL.

[30] Figure 9. Sequence, host string content, and structure of L2 AC10 VL.

[31] Figure 10. Sequence, host string content, and structure of L3 AC10 VL.

[32] Figure 11. Sequence, host string content, and structure of H1 AC10 VH.

[33] Figure 12. Sequence, host string content, and structure of H2 AC10 VH.

[34] Figure 13. Sequence, host string content, and structure of H3 AC10 VH.

[35] Figures 14. AlphaScreen™ assay measuring binding between AC10 variants and the target antigen CD30. In the presence of competitor variant antibody, a characteristic inhibition curve is observed as a decrease in luminescence signal. The binding data were normalized to the maximum and minimum luminescence signal for each particular curve, provided by the baselines at low and high antibody concentrations respectively. The curves represent the fits of the data to a one site competition model using nonlinear regression, and the fits provide IC50s for each antibody.

[36] Figure 15. Figure 11. SPR sensorgrams showing binding of AC10 WT and variant full length antibodies to the CD30 target antigen. The curves consist of an association phase and dissociation phase, the separation being marked by a little spike on each curve.

[37] Figure 16. AlphaScreen™ assay measuring binding between AC10 variants and human V158 FcγRIIIa.

[38] Figure 17. Cell-based ADCC assay of WT and AC10 variants. Purified human peripheral blood monocytes (PBMCs) were used as effector cells, L540 Hodgkin's lymphoma cells were used as target cells, and lysis was monitored by measuring LDH activity using the Cytotoxicity Detection Kit (LDH, Roche Diagnostic Corporation, Indianapolis, IN). Samples were run in triplicate to provide error estimates (n=3, +/- S.D.). Figure 17 shows the dose dependence of ADCC at various antibody concentrations, and the curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression. Raw data are presented in Figures 17a and 17b, whereas in Figure 17c the data were normalized to a percentage scale of maximal cytotoxicity determined by Triton-X100 lysis of target cells.

[39] Figure 18. Cell-based assay measuring ADCC capacity of WT (H0/L0) and H3/L3 AC10 antibodies comprising Fc variants that provide enhanced effector function. Raw data were normalized to a percentage scale of maximal cytotoxicity determined by Triton-X100 lysis of target cells.

[40] Figure 19. AlphaScreen™ assay measuring binding between select H3L3 secondary AC10 variants and the target antigen CD30.

[41] Figure 20. Sequence, host string content, and structure of L3.71 AC10 VL.

[42] Figure 21. Sequence, host string content, and structure of L3.72 AC10 VL.

[43] Figure 22. Sequence, host string content, and structure of H3.68 AC10 VH.

[44] Figure 23. Sequence, host string content, and structure of H3.69 AC10 VH.

[45] Figure 24. Sequence, host string content, and structure of H3.70 AC10 VH.

[46] Figure 25. Amino acid sequences of a AC10 variant antibodies comprising the L3.71 AC10 variant VL with the CL□ constant light chain (Figure 25a) and the H3.70 AC10 variant VH with IgG constant chains (Figures 25b – 25e) that may comprise amino acid modifications in the Fc region. Figure 25b provides an IgG1 heavy chain with positions that may be mutated designated in bold as X_1, X_2, X_3, and X_4, referring to residues S239, V264, A330, and I332. Figure 25c provides one example of a heavy chain described in Figure 25b, here comprising the H3.70 AC10 variant VH region with the S239D/A330L/I332E IgG1 constant region. Figure 25d provides an IgG2 heavy chain with positions that may be mutated and designated in bold as X_1, X_2, X_3, X_4, Z_1, Z_2, Z_3, Z_4, and Z_5 referring to residues S239, V264, A330, I332, P233, V234, A235, -236, and G237 (here –236 refers to a deletion at EU index position 236). Figure 25e provides one example of a heavy chain described in Figure 25d, here comprising the H3.70 AC10 variant VH region with the S239D/A330L/I332E/P233E/V234L/A235L/-236G IgG2 constant region.

[47] Figure 26. Sequence, host string content, and structure of WT C225 VL.

[48] Figure 27. Sequence, host string content, and structure of WT C225 VH.

[49] Figure 28. Sequence, host string content, and structure of CDR grafted C225 VL, which was derived from the CDRs of WT C225 and the frameworks of the human germline sequence vlk_6D-21 and an L106I (Kabat numbering) substitution in FR4.

[50] Figure 29. Sequence, host string content, and structure of CDR grafted C225 VH, which was derived from the CDRs of WT C225 and the frameworks of the human germline sequence vh_4-30-4 and an A113S (Kabat numbering) substitution in FR4.

[51] Figure 30. C225 VL and VH variants with optimized HSC. The nonredundant set of output sequences from the calculations described in Example 2 are shown.

[52] Figure 31. C225 VL and VH variants with optimized HSC. The nonredundant set of output sequences from the calculations described in Example 2 are shown.

[53] Figure 32. Sequence, host string content, and structure of L2 C225 VL.

[54] Figure 33. Sequence, host string content, and structure of L3 C225 VL.

[55] Figure 34. Sequence, host string content, and structure of L4 C225 VL.

[56] Figure 35. Sequence, host string content, and structure of H3 C225 VH.

[57] Figure 36. Sequence, host string content, and structure of H4 C225 VH.

[58] Figure 37. Sequence, host string content, and structure of H5 C225 VH.

[59] Figure 38. Sequence, host string content, and structure of H6 C225 VH.

[60] Figure 39. Sequence, host string content, and structure of H7 C225 VH.

[61] Figure 40. Sequence, host string content, and structure of H8 C225 VH.

[62] Figure 41. SPR sensorgrams showing binding of full length antibody C225 variants to the EGFR target antigen. The sensorgrams show binding of C225 WT (L0/H0) and variant (L0/H3, L0/H4, L0/H5, L0/H6, L0/H7, L0/H8, L2/H3, L2/H4, L2/H5, L2/H6, L2/H7, L2/H8, L3/H3, L3/H4, L3/H5, L3/H6, L3/H7, L3/H8, L4/H3, L4/H4, L4/H5, L4/H6, L4/H7, and L4/H8) full length antibodies to the EGFR sensor chip. The curves consist of an association phase and dissociation phase, the separation being marked by a little spike on each curve.

[63] Figures 42. Cell-based ADCC assay of C225 WT (L0/H0) and variant (L0/H3, L0/H4, L0/H5, L0/H6, L0/H7, L0/H8, L2/H3, L2/H4, L2/H5, L2/H6, L2/H7, L2/H8, L3/H3, L3/H4, L3/H5, L3/H6, L3/H7, L3/H8, L4/H3, L4/H4, L4/H5, L4/H6, L4/H7, and L4/H8) full length antibodies. Purified human peripheral blood monocytes (PBMCs) were used as effector cells, A431 epidermoid carcinoma cells were used as target cells at a 10:1 effector:target cell ratio, and lysis was monitored by measuring LDH activity using the Cytotoxicity Detection Kit (LDH, Roche Diagnostic Corporation, Indianapolis, IN). Samples were run in triplicate to provide error estimates (n=3, +/- S.D.). Figure 42 shows the dose dependence of ADCC at various antibody concentrations, normalized to the minimum and maximum levels of lysis for the assay. The curves represent the fits of the data to a sigmoidal dose-response model using nonlinear regression.

[64] Figure 43. Sequence, host string content, and structure of WT ICR62 VL.

[65] Figure 44. Sequence, host string content, and structure of WT ICR62 VH.

[66] Figure 45. Sequence, host string content, and structure of CDR grafted ICR62 VL. CDR grafted ICR62 VL was derived from the CDRs of WT ICR62 and the frameworks of the human germline sequence vlk_1-17 and an L106I (Kabat numbering) substitution in FR4.

[67] Figure 46. Sequence, host string content, and structure of CDR grafted ICR62 VH. CDR grafted ICR62 VH was derived from the CDRs of WT ICR62 and the frameworks of the human germline sequence vh_1-f and substitutions A107T and S108L (Kabat numbering) in FR4.

[68] Figure 47. ICR62 VL and VH variants with optimized HSC.

[69] Figure 48. Sequence, host string content, and structure of L3 ICR62 VL.

[70] Figure 49. Sequence, host string content, and structure of H9 ICR62 VH.

[71] Figure 50. Sequence, host string content, and structure of H10 ICR62 VH.

[72] Figure 51. Comparison of VH sequences humanized by the methods in the prior art versus the present method. Prior art antibodies include Ctm01, A5B7, Zenapax, MaE11, 1129, MHM2, H52, Huzaf, Hu3S193, D3H44, AQC2, 2C4, D3H44, Hfe7A, 5C8, m4D5, A.4.6.1, Campath, HuLys11, A.4.6.1, Mylotarg, MEDI-507, huH65_vh, EP-5C7, 9F3, HPC4, 38C2, Br96, 1A6, and 6.7. Sequences designed using the present invention, including AC10 H1, H2, and H3, C225 H3, H4, H5, H6, H7, and H8, and ICR62 H9 and H10, are offset to the right. Figure 51a provides the host string content (HSC) as defined by equation 3, Figure 51b provides the exact string content (ESC) as defined by equation 3a, and Figure 51c provides the framework region homogeneity (FRH) as defined by equation 10. Window size w was 9 for all calculations.

[73] Figure 52. Comparison of VL sequences humanized by the methods in the prior art versus the present method. Prior art antibodies include Ctm01, A5B7, Zenapax, MaE11, 1129, MHM2, H52, Huzaf, Hu3S193, D3H44, AQC2, 2C4, D3H44, Hfe7A, 5C8, m4D5, A.4.6.1, Campath, HuLys11, A.4.6.1, Mylotarg, MEDI-507, huH65_vh, EP-5C7, 9F3, HPC4, 38C2, Br96, 1A6, and 6.7. Sequences designed using the present invention, including AC10 L1, L2, and L3, C225 L2, L3, L4, and ICR62 L2, are offset to the right. Figure 52a provides the host string content (HSC) as defined by equation 3, Figure 52b provides the exact string content (ESC) as defined by equation 3a, and Figure 52c provides the framework region homogeneity (FRH) as defined by equation 10. Window size w was 9 for all calculations.

DETAILED DESCRIPTION OF THE INVENTION

Definitions

[74] In order that the invention may be more completely understood, several definitions are set forth below. Such definitions are meant to encompass grammatical equivalents.

[75] By "amino acid" as used herein is meant one of the 20 naturally occurring amino acids or any non-natural analogues that may be present at a specific, defined position.

[76] By "amino acid modification" herein is meant an amino acid substitution, insertion, and/or deletion in a polypeptide sequence. The preferred amino acid modification herein is a substitution.

[77] By "amino acid substitution" or "substitution" herein is meant the replacement of an amino acid at a given position in a protein sequence with another amino acid.

[78] By "antibody" herein is meant a protein consisting of one or more proteins substantially encoded by all or part of the recognized immunoglobulin genes. The recognized immunoglobulin genes, for example in humans, include the kappa (κ), lambda (λ), and heavy chain genetic loci, which together comprise the myriad variable region genes, and the constant region genes mu (μ), delta (δ), gamma (γ), sigma (σ), and alpha (α) which encode the IgM, IgD, IgG, IgE, and IgA isotypes respectively. Antibody herein is meant to include full length antibodies and antibody fragments, and may refer to a natural antibody from any organism, an engineered antibody, or an antibody generated recombinantly for experimental, therapeutic, or other purposes. By "IgG" as used herein is meant a protein belonging to the class of antibodies that are substantially encoded by a recognized immunoglobulin gamma gene. In humans this class comprises IgG1, IgG2, IgG3, and IgG4.

[79] By "corresponding" or "equivalent" residues as meant herein are residues that represent similar or homologous sequence and/or structural environments between a first and second protein, or between a first protein and set of multiple proteins. In order to establish homology, the amino acid sequence of a first protein is directly compared to the sequence of a second protein. After aligning the sequences, using one or more of the homology alignment programs known in the art (for example using conserved residues as between species), allowing for necessary insertions and deletions in order to maintain alignment (i.e., avoiding the elimination of conserved residues through arbitrary deletion and insertion), the residues equivalent to particular amino acids in the primary sequence of the first protein are defined. Alignment of conserved residues preferably should conserve 100% of such residues. However, alignment of greater than 75% or as little as 50% of conserved residues is also adequate to define equivalent residues. Corresponding residues may also

be defined by determining structural homology between a first and second protein that is at the level of tertiary structure for proteins whose structures have been determined. In this case, equivalent residues are defined as those for which the atomic coordinates of two or more of the main chain atoms of a particular amino acid residue of the proteins (N on N, CA on CA, C on C and O on O) are within 0.13 nm and preferably 0.1 nm of each other after alignment. Alignment is achieved after the best model has been oriented and positioned to give the maximum overlap of atomic coordinates of non-hydrogen protein atoms of the proteins.

[80] By "CDR" as used herein is meant a Complementarity Determining Region of an antibody variable domain. Systematic identification of residues included in the CDRs have been developed by Kabat (Kabat et al., 1991, Sequences of Proteins of Immunological Interest, 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda) and alternately by Chothia (Chothia & Lesk, 1987, J. Mol. Biol. 196: 901-917; Chothia et al., 1989, Nature 342: 877-883; Al-Lazikani et al., 1997, J. Mol. Biol. 273: 927-948). For the purposes of the present invention, CDRs are defined as a slightly smaller set of residues than the CDRs defined by Chothia. VL CDRs are herein defined to include residues at positions 27-32 (CDR1), 50-56 (CDR2), and 91-97 (CDR3), wherein the numbering is according to Chothia. Because the VL CDRs as defined by Chothia and Kabat are identical, the numbering of these VL CDR positions is also according to Kabat. VH CDRs are herein defined to include residues at positions 27-33 (CDR1), 52-56 (CDR2), and 95-102 (CDR3), wherein the numbering is according to Chothia. These VH CDR positions correspond to Kabat positions 27-35 (CDR1), 52-56 (CDR2), and 95-102 (CDR3).

[81] By "framework" as used herein is meant the region of an antibody variable domain exclusive of those regions defined as CDR's. Each antibody variable domain framework can be further subdivided into the contiguous regions separated by the CDR's (FR1, FR2, FR3 and FR4).

[82] By "germline" as used herein is meant the set of sequences that compose the natural genetic repertoire of a protein, and its associated alleles.

[83] By "host" as used herein is meant a family, genus, species or subspecies, group of individuals or even a single individual. A host group of individuals can be selected for based upon a variety of criteria, such as MHC allele composition, etc. In a preferred embodiment, a host is canine, murine, primate, or human. In the most preferred embodiment, a host is human.

[84] By "host string" or "host sequence" as used herein is meant a string or sequence that

encodes any part of a naturally occurring host protein.

[85] By "humanized" antibody as used herein is meant an antibody comprising a human framework region and one or more CDR's from a non-human (usually mouse or rat) antibody. The non-human antibody providing the CDR's is called the "donor" and the human immunoglobulin providing the framework is called the "acceptor". One says that the donor antibody has been "humanized", by the process of "humanization".

[86] By "identity" as used herein is meant the number of residues in a first sequence that are identical to the residues in a second sequence after alignment of the sequences to achieve the maximum identity.

[87] By "immune epitope" or "epitope" herein is meant a linear sequence of amino acids that is located in a protein of interest. Epitopes may be analyzed for their potential for immunogenicity. Epitopes may be any length, preferably 9-mers.

[88] By "immunogenicity" herein is meant the ability of a protein to elicit an immune response, including but not limited to production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, and anaphylaxis.

[89] By "immunoglobulin (Ig)" herein is meant a protein consisting of one or more proteins substantially encoded by immunoglobulin genes. Immunoglobulins include but are not limited to antibodies. Immunoglobulins may have a number of structural forms, including but not limited to full length antibodies, antibody fragments, and individual immunoglobulin domains. By "immunoglobulin (Ig) domain" herein is meant a region of an immunoglobulin that exists as a distinct structural entity as ascertained by one skilled in the art of protein structure. Ig domains typically have a characteristic β-sandwich folding topology. The known Ig domains in the IgG class of antibodies are V_H, CH1 ($C\gamma1$), CH2 ($C\gamma2$), CH3 ($C\gamma3$), V_L, and C_L.

[90] By "natural sequence" or "natural protein" as used herein is meant a protein that has been determined to exist absent any experimental modifications. Also included are sequences that can be predicted to exist in nature based on experimentally determined sequences. An example of such a predicted sequence is an antibody that can be predicted to exist based on the established patterns of germline recombination. In this case the large size of the predicted antibody repertoire makes the actual experimental determination of all mature recombined antibodies not practical.

[91] By "parent" or "parent protein" as used herein is meant a protein that is subsequently

modified to generate a variant. The parent protein may be a naturally occurring protein, or a variant or engineered version of a naturally occurring protein. Parent protein may refer to the protein itself, compositions that comprise the parent protein, or the amino acid sequence that encodes it. Accordingly, by "parent antibody" as used herein is meant an antibody that is subsequently modified to generate a variant antibody. Accordingly, by "parent sequence" as used herein is meant the sequence that encodes the parent protein or parent antibody.

[92] By "position" as used herein is meant a location in the sequence of a protein. Positions may be numbered sequentially, or according to an established format, for example Kabat, Chothia, and/or the EU index as in Kabat.

[93] By "protein" herein is meant at least two covalently attached amino acids, which includes proteins, polypeptides, oligopeptides and peptides. The protein may be made up of naturally occurring amino acids and peptide bonds, or synthetic peptidomimetic structures.

[94] By "reduced immunogenicity" herein is meant a decreased ability to activate the immune system, when compared to the parent protein. For example, a protein variant can be said to have "reduced immunogenicity" if it elicits neutralizing or non-neutralizing antibodies in lower titer or in fewer patients than the parent protein. A protein variant also can be said to have "reduced immunogenicity" if it shows decreased binding to one or more MHC alleles or if it induces T cell activation in a decreased fraction of patients relative to the parent protein.

[95] By "residue" as used herein is meant a position in a protein and its associated amino acid identity. For example, proline 9 (also referred to as Pro9, also referred to as P9) is a residue in the WT AC10 VH region.

[96] By "scoring function" herein is meant any equation or method for evaluating the fitness of one or more amino acid modifications in a protein. The scoring function may involve a physical or chemical energy term, or may involve knowledge-, statistical-, sequence-based energy terms, and the like.

[97] By "string" as used herein is meant a contiguous sequence that encodes any part of a protein. Strings may comprise any 2 or more linear residues, with the number of contiguous residues being defined by the "window" or "window size". Window sizes of 2 - 20 are preferred, with 7 - 13 more preferred, with 9 most preferred.

[98] By "target" as used herein is meant the molecule that is bound specifically by a protein. A target may be a protein, carbohydrate, lipid, or other chemical compound. The target of an antibody is its antigen, also referred to as its target antigen.

[99] By "variable region" as used herein is meant the region of an immunoglobulin that comprises one or more Ig domains substantially encoded by any of the VL (including $V\kappa$ and $V\lambda$) and/or V_H genes that make up the light chain (including kappa and lambda) and heavy chain immunoglobulin genetic loci respectively. A light or heavy chain variable region (VL and VH) consists of a "framework" or "FR" region interrupted by three hypervariable regions referred to as "complementarity determining regions" or "CDRs". The extent of the framework region and CDRs have been precisely defined, for example as in Kabat (see "Sequences of Proteins of Immunological Interest," E. Kabat et al., U.S. Department of Health and Human Services, (1983)), and as in Chothia. The framework regions of an antibody, that is the combined framework regions of the constituent light and heavy chains, serves to position and align the CDRs, which are primarily responsible for binding to an antigen.

[100] By "variant protein" or "protein variant", or "variant" as used herein is meant a protein that differs from a parent protein by virtue of at least one amino acid modification. Protein variant may refer to the protein itself, a composition comprising the protein, or the amino sequence that encodes it. Preferably, the protein variant has at least one amino acid modification compared to the parent protein, e.g. from about one to about ten amino acid modifications, and preferably from about one to about five amino acid modifications compared to the parent. The protein variant sequence herein will preferably possess at least about 80% homology with a parent protein sequence, and most preferably at least about 90% homology, more preferably at least about 95% homology. Accordingly, by "immunoglobulin variant" as used herein is meant an immunoglobulin that differs from a parent immunoglobulin by virtue of at least one amino acid modification.

[101] By "wild type or WT" herein is meant an amino acid sequence or a nucleotide sequence that is found in nature and includes allelic variations. A WT protein has an amino acid sequence or a nucleotide sequence that has not been intentionally modified.

[102] The protein variants of the present invention may be derived from parent proteins that are themselves from a wide range of sources. The parent protein may be substantially encoded by one or more genes from any organism, including but not limited to humans, mice, rats, rabbits, camels, llamas, dromedaries, monkeys, preferably mammals and most preferably humans and mice and rats. Although in a preferred embodiment the parent protein is nonhuman, in some embodiments of the present invention the parent protein may be human or similar to human. The parent protein may comprise more than one protein chain, and thus may be a monomer or an oligomer, including a homo- or hetero-oligomer. In

a preferred embodiment, the parent protein is an antibody, referred to as the parent antibody. The parent antibody need not be naturally occurring. For example, the parent antibody may be an engineered antibody, including but not limited to nonhuman and chimeric antibodies. The parent antibody may be fully human, obtained for example using transgenic mice (Bruggemann *et al.*, 1997, *Curr Opin Biotechnol* 8:455-458) or human antibody libraries coupled with selection methods (Griffiths *et al.*, 1998, *Curr Opin Biotechnol* 9:102-108). The parent antibody need not be naturally occurring. For example, the parent antibody may be an engineered antibody, including but not limited to chimeric antibodies and humanized antibodies (Clark, 2000, *Immunol Today* 21:397-402). The parent antibody may be an engineered variant of an antibody that is substantially encoded by one or more natural antibody genes. In one embodiment, the parent antibody has been affinity matured, as is known in the art, or engineered in some other way. The parent antibodies of the present invention may be substantially encoded by immunoglobulin genes belonging to any of the antibody classes, and may comprise sequences belonging to the IgG class of antibodies, including IgG1, IgG2, IgG3, or IgG4, or alternatively the IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG, or IgM classes of antibodies.

[103] Virtually any binding partner or antigen may be targeted by the proteins of the present invention. A number biotherapeutic proteins and antibodies that are approved for use, in clinical trials, or in development may thus benefit from immunogenicity reduction methods of the present invention. In a preferred embodiment, the less immunogenic protein of the present invention is an antibody. The less immunogenic antibody may comprise sequences belonging to the IgG (including IgG1, IgG2, IgG3, or IgG4), IgA (including subclasses IgA1 and IgA2), IgD, IgE, IgG, or IgM classes of antibodies, with the IgG class being preferred. The less immunogenic antibodies of the present invention may be full length antibodies, or antibody fragments. Constant regions need not be present, but if they are, they will likely be substantially identical to human immunoglobulin constant regions.

[104] The constant region of the antibody may be modified in some way to make it more effective therapeutically. For example, the constant region may comprise substitutions that enhance therapeutic properties. Most preferred substitutions and optimized effector function properties are described in USSN 10/672,280, PCT US03/30249, and USSN 10/822,231, and USSN 60/627,774, filed 11/12/2004 and entitled "Optimized Fc Variants". Other known Fc variants that may find use in the present invention include but are not limited to those described in US 6,737,056; PCT US2004/000643; USSN 10/370,749; PCT/US2004/005112; US 2004/0132101; USSN 10/672,280; PCT/US03/30249; US 6,737,056, US 2004/0002587; WO 2004/063351; Idusogie et al., 2001, J. Immunology 166:2571-2572; Hinton et al., 2004,

J. Biol. Chem. 279(8): 6213-6216. In alternate embodiments, the constant region may comprise one or more engineered glycoforms, as is known in the art (Umaña *et al.*, 1999, *Nat Biotechnol* 17:176-180; Davies *et al.*, 2001, *Biotechnol Bioeng* 74:288-294; Shields *et al.*, 2002, *J Biol Chem* 277:26733-26740; Shinkawa *et al.*, 2003, *J Biol Chem* 278:3466-3473); (US 6,602,684; USSN 10/277,370; USSN 10/113,929; PCT WO 00/61739A1; PCT WO 01/29246A1; PCT WO 02/31140A1; PCT WO 02/30954A1); (Potelligent™ technology [Biowa, Inc., Princeton, NJ]; GlycoMAb™ glycosylation engineering technology [GLYCART biotechnology AG, Zürich, Switzerland]).

[105] The protein variants of the present invention may find use in a wide range of protein products. In one embodiment the protein is a therapeutic, a diagnostic, or a research reagent, preferably a therapeutic. Alternatively, the protein of the present invention may be used for agricultural or industrial uses. In a preferred embodiment, the protein is a therapeutic that is used to treat a disease. By "disease" herein is meant a disorder that may be ameliorated by the administration of a pharmaceutical composition comprising a protein of the present invention. Diseases include but are not limited to autoimmune diseases, immunological diseases, infectious diseases, inflammatory diseases, neurological diseases, and oncological and neoplastic diseases including cancer. In one embodiment, a protein of the present invention is the only therapeutically active agent administered to a patient. Alternatively, the protein of the present invention is administered in combination with one or more other therapeutic agents, including but not limited to cytotoxic agents, chemotherapeutic agents, cytokines, growth inhibitory agents, anti-hormonal agents, kinase inhibitors, anti-angiogenic agents, cardioprotectants, or other therapeutic agents. The proteins of the present invention may be combined with other therapeutic regimens. For example, in one embodiment, the patient to be treated with the protein may also receive radiation therapy and/or undergo surgery. In an alternate embodiment, the protein of the present invention is conjugated or operably linked to another therapeutic compound. The therapeutic compound may be a cytotoxic agent, a chemotherapeutic agent, a toxin, a radioisotope, a cytokine, or other therapeutically active agent. In yet another embodiment, a protein of the present invention may be conjugated to a protein or molecule for utilization in tumor pretargeting or prodrug therapy. Other modifications of the proteins of the present invention are contemplated herein. For example, the protein may be linked to one of a variety of nonproteinaceous polymers, for example e.g., polyethylene glycol (PEG).

[106] Pharmaceutical compositions are contemplated wherein a protein of the present invention and one or more therapeutically active agents are formulated. Formulations of the proteins of the present invention are prepared for storage by mixing the protein having the

desired degree of purity with optional pharmaceutically acceptable carriers, excipients or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed.,1980), in the form of lyophilized formulations or aqueous solutions. The formulations to be used for *in vivo* administration are preferably sterile. The proteins disclosed herein may also be formulated as immunoliposomes, or entrapped in microcapsules. The concentration of the protein of the present invention in the formulation may vary from about 0.1 to 100 weight %. In a preferred embodiment, the concentration of the protein is in the range of 0.003 to 1.0 molar. In order to treat a patient, a therapeutically effective dose of the protein of the present invention may be administered. The exact dose will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques. Dosages may range from 0.01 to 100 mg/kg of body weight or greater, for example 0.1, 1, 10, or 50 mg/kg of body weight, with 1 to 10mg/kg being preferred. Administration of the pharmaceutical composition comprising a protein of the present invention, preferably in the form of a sterile aqueous solution, may be done in a variety of ways, including, but not limited to, orally, subcutaneously, intravenously, intranasally, intraotically, transdermally, topically, intraperitoneally, intramuscularly, intrapulmonary, inhalably, vaginally, parenterally, rectally, or intraocularly. As is known in the art, the pharmaceutical composition may be formulated accordingly depending upon the manner of introduction.

Description of the Methodology

[107] The present invention provides a novel method for reducing the immunogenicity of a protein. A central principle of the described method is that substitutions are designed to maximize the content of human linear sequence strings using an alignment of human sequences. For application to antibodies, this approach to immunogenicity reduction excludes the use of the single donor-acceptor model employed in humanization methods. By stepping outside of the limitations imposed by the need to choose a human acceptor sequence a priori, a more immunologically relevant approach to immunogenicity reduction is enabled. Sequence information and structural information may be used to score potential amino acid substitutions. The scoring results are used to design protein variant libraries, which are subsequently screened experimentally to determine favorable substitutions. Feedback from experimental data may guide subsequent iterations of design and experimental screening, ultimately enabling protein variants to be engineered with the optimal balance between biophysical and immunological constraints.

Sequences

[108] Central to the method described herein is that a set of host sequences provides

information as to the degree to which linear sequence strings have the potential to be immunogenic. Thus the set of sequences employed is an important parameter. In the most common embodiment, the sequences are a set of human sequences that are homologous in sequence and/or structure to the parent sequence. As is known in the art, some proteins share a common structural scaffold and are homologous in sequence. This information may be used to gain insight into particular positions in the protein family. Sequence alignments are often carried out to determine which protein residues are conserved and which are not conserved. That is to say, by comparing and contrasting alignments of protein sequences, the degree of variability at a position may be observed, and the types of amino acids that occur naturally at positions may be observed. Thus for the present invention, typically the sequences are aligned such that the conserved or similar residues that exist between the parent sequence and the set of human sequences and among the set of human sequences can be identified. Methods for sequence alignment are well known in the art, and include alignments based on sequence and structural homology.

[109] Protein sequence information can be obtained, compiled, and/or generated from sequence alignments of naturally occurring proteins from any organism, including but not limited to mammals. Because a preferred embodiment of present invention is directed towards immunogenicity reduction for biotherapeutics, the sequences that compose the set are most preferably human. The source of the sequences may vary widely, may be a database that is compiled publicly or privately, and may be may include one or more of the known general protein and nucleic acid sequences databases, including but not limited to SwissProt, GenBank and Entrez, and EMBL Nucleotide Sequence Database. Because a preferred embodiment of the present invention is its application to the immunogenicity reduction of immunoglobulins, a number of immunoglobulin databases may be useful for obtaining sequences, including but not limited to the Kabat database (Johnson & Wu, 2001, *Nucleic Acids Res* 29:205-206; Johnson & Wu, 2000, *Nucleic Acids Res* 28:214-218), the IMGT database (IMGT, the international ImMunoGeneTics information system®; Lefranc *et al.*, 1999, *Nucleic Acids Res* 27:209-212; Ruiz *et al.*, 2000 *Nucleic Acids Re.* 28:219-221; Lefranc *et al.*, 2001, *Nucleic Acids Res* 29:207-209; Lefranc *et al.*, 2003, *Nucleic Acids Res* 31:307-310), and VBASE.

[110] As is well known in the art, immunoglobulins possess a high degree of sequence and structural homology, and therefore alignment of sequences provides a wealth of information. Due to the existence of deletions and insertions in these alignments, numbering conventions have been adopted to enable a normalized reference to conserved positions in immunoglobulin families or subfamilies. Those skilled in the art will appreciate that these

conventions consist of nonsequential numbering in specific regions of an immunoglobulin sequence, and thus accordingly the positions of any given immunoglobulin as defined by any given numbering scheme will not necessarily correspond to its sequential sequence or to those in an alternate numbering scheme. For all variable regions discussed in the present invention, numbering is according to the numbering scheme of Kabat (Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest,* 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda). For all constant region positions discussed in the present invention, number is according to the EU index as in Kabat. Alternate numbering schemes may find use in the present invention, including but not limited that of Chothia (Chothia & Lesk, 1987, *J. Mol. Biol.* 196: 901-917; Chothia *et al.*, 1989, *Nature* 342: 877-883; Al-Lazikani *et al.*, 1997, *J. Mol. Biol.* 273: 927-948).

[111] In a most preferred embodiment, the set of human sequences used is an aligned set of human germline immunoglobulin sequences. For example, Figures 1a – 1c provide the set of sequences that compose the human antibody variable region germline (VH, VL, and J chains), along with the corresponding diversity at each position. The human germline repertoire for immunoglobulin heavy chain variable regions and immunoglobulin light chain kappa variable regions have been reported (Matsuda et al., 1998, *J Exp Med* 188: 2151–2162; Zachau, 2000, Biol Chem 381:951–954; Pallares et al., 1999, *Exp Clin Immunogenet* 16(1): 36-60; Barbie & Lefranc, 1998, *Exp Clin Immunogenet* 15(3): 171-83). The human immunoglobulin kappa variable (IGKV) genes and joining (IGKJ) segments. Barbie V, Lefranc MP). The rationale for use of this type of sequence information as a metric for humanness is that the strings that compose the human germline should be minimally immunogenic. Sequences need not be human genomic or germline sequences. In other preferred embodiments, human antibody variable region sequences are derived not from germline information, but rather from matured antibodies obtained for example from hybridoma technology or cDNA libraries.

[112] For many of the genes in the human immunoglobulin germline, several different alleles have been identified. Although the polymorphisms detected in many of the alleles do not change the amino acid sequence of the gene, in a great number of cases the sequence is changed. In choosing a set of sequences to use in the method described herein, different sets of sequences may be chosen. When choosing a single allele as representative of a specific gene the most cautious approach is to choose that sequence which is closest to the consensus of the entire germline. This subset of sequences would thereby be most likely to be represented within the population as a whole. Alternatively, a much greater sequence diversity could be sampled by choosing representative sequences that are furthest from the

consensus. Another approach yielding greater diversity would be to use multiple alleles where they exist for each germline. At this time, there is little or no quantitative data on allele frequency within the population. When allele frequency becomes available, a more informed decision can be made regarding the likelihood of tolerance for a specific non-consensus allele within the target patient population.

[113] When two or more possible substitutions are being evaluated for use at a specific position when both are found in the human germline, the decision may become subjective. In such a case additional information can be incorporated that may reflect different levels of expression of particular genes (Cox *et al.* Eur J Immunol. 1994 Apr;24(4):827-36). One underlying assumption of such a strategy would be that relative expression level of a particular germline (or corresponding sequence strings) correlates with the relative immunogenicity.

[114] The sequences used for the method disclosed herein are those of homologous proteins with sufficient homology to allow their alignment with the protein whose immunogenicity is being reduced. One might argue that if a particular protein sequence is found anywhere within the expressed human genome that there is innate tolerance to that peptide. Such a proposition greatly increases the number of possible sequences that could be used to reduce the immunogenicity of a protein. In such a case however, alignment of proteins that are not structurally homologous would likely be prohibitive. In addition, the processing of a protein to produce the strings to which tolerance is developed may be structurally determined. Therefore, a specific strings may be nonimmunogenic in its native context but immunogenic in an altered structural context.

Scoring Functions – String Content

[115] In order to evaluate the fitness of protein variants, amino acid modifications in the parent protein may be scored using a variety of scoring functions. Central to preferred embodiment of immunogenicity reduction method described herein is that at least one scoring function is aimed at maximizing the content of host linear sequence strings that are present in a set of host sequences. Typically, but not always, a computer is used to score potential amino acid substitutions.

[116] In one embodiment, substitutions may be scored according to their occupancy in the set of host sequences, i.e., whether or not a given amino acid is part of the diversity at a given position. The use of position-specific alignment information to generate a list of considered amino acids at a variable position is well known in the art; see for example Lehmann & Wyss, 2001, *Curr Opin Biotechnol* 12(4): 371-5; Lehmann *et al.*, 2000, *Biochim*

Biophys Acta 1543(2):408-415; Rath & Davidson, 2000, *Protein Sci*, 9(12):2457-69; Lehmann *et al.*, 2000, *Protein Eng* 13(1):49-57; Desjarlais & Berg, 1993, *Proc Natl Acad Sci USA* 90(6):2256-60; Desjarlais & Berg, 1992, *Proteins* 12(2):101-4; Henikoff & Henikoff, 2000, *Adv Protein Chem* 54:73-97; Henikoff & Henikoff, 1994, *J Mol Biol* 243(4):574-8. Thus, for example, for the parent nonhuman VLκ sequence aligned to the human sequences in Figure 1b, substitutions to be considered at position 1 would be Ala, Asp, Glu, Asn, and Val. In a more preferred embodiment, substitutions are scored based on their frequency in the set of human sequences listed. For example, in the previous example, Asp and Glu occur most frequently at position 1, and thus may be more preferable substitutions that Ala, Asn, or Val. The basis for this scoring function is that the frequency of a given amino acid at a given position in the alignment is proportional to its potential for being in a host string.

[117] Occupancy and frequency provide relatively straightforward approximations for designing substitutions that have the potential for reduced immunogenicity. Their use, however, does not take into account the context of the parent sequence. Although frequency is proportional to the potential for a substitution to increase host content of a string, it is not a direct measure. In order to more accurately incorporate the information present in an aligned set of host sequences into a measure of immunogenicity, an approach can be taken wherein the linearity or contiguity of a given position in the context of the strings that comprise it is considered. In this most preferred embodiment, substitutions in a parent sequence are scored based on the probability of removing a nonhost string and replacing it with a less immunogenic string, namely one present in the set of host sequences. This method of scoring may employ the calculation of identity or percent identity of a parent string to a host string within a window of equivalent positions. In one embodiment, the identity of a string in sequence s to a host string in sequence h, (IDstring), can be presented as the sum of amino acid sequence identities in a given window size, according to equation 1:

Equation 1 $$IDstring(i,w) = \sum_{j=i}^{i+w-1} \delta_{aa_j^s, aa_j^h}$$

where w is the string window size, i is the first position in the string, aa^s_j is the amino acid at position j of sequence s, aa^h_j is the amino acid at position j of the host sequence h, and the Kronecker delta function is used to return a value of 1 for a match (for example if the parent and host amino acids at position j are both serine) and 0 if there is no match (for example if the parent amino acid at position j is a serine but the host amino acid is a leucine). Figure 2a illustrates equation 1 using a region of the VH of murine anti-Her2 antibody m4D5

(VH_m4D5) as the parent sequence s and the homologous region from the VH human germline (VH_1-2) as human sequence h.

[118] In a further embodiment, it is assumed that the most immunologically appropriate measure of host string content at position i is the maximal identity between a string of sequence s and any host sequence in the alignment, as calculated in equation 2:

Equation 2 $$ID\max = \max_{h \in HS}\left(\sum_{j=i}^{i+w-1} \delta_{aa_j^s, aa_j^h}\right)$$

where HS is the set of host sequences. In other words, if IDstring at position i is equal to w for any one of the host sequences, IDmax = w as well, and the i^{th} string is assumed to be minimally immunogenic. The concept of the IDmax quantity represented by Equation 2 is illustrated in Figure 2b.

[119] Finally, these equations can be combined to calculate a single numerical metric for total host string content (HSC) of a sequence s by summing the IDmax values over all pertinent sequence positions, as in equation 3:

Equation 3 $$HSC(s) = 100 \cdot \frac{1}{(L-w+1)\cdot w} \sum_{i=1}^{L-w+1} \max_{h \in HS}\left(\sum_{j=i}^{i+w-1} \delta_{aa_j^s, aa_j^h}\right)$$

where L is the length of the sequence and HS is the set of host sequences in the alignment. A perfectly host sequence would have an HSC of 100. One might alternatively say that such a sequence is 100% host. The concept of the HSC quantity represented by Equation 3 is illustrated in Figure 2c. In alternative embodiments, Equation 3 can be modified further such that the final score is dependent on the relative usage of each host sequence in the alignment. Strings from sequences that are more frequently expressed by hosts are expected to be more tolerized, and therefore may be given correspondingly higher influence in a scoring system.

[120] In an alternative embodiment, one can measure the exact string content (ESC) as in Equation 3a:

Equation 3a $$ESC(s) = 100 \cdot \frac{1}{(L-w+1)} \sum_{i=1}^{L-w+1} \max_{h \in HS} \delta_{aa_{i..i+w-1}^s, aa_{i..i+w-1}^h},$$

where the notation $aa^s_{i..i+w-1}$ refers the contiguous sequence string in protein s from position i to position i+w-1. In this embodiment, only perfect matches of size w are counted in the score.

[121] It is worth noting that, since the scoring systems in Equations 3 and 3a are based on

local sequence identity and/or similarity evaluated over windows of defined size, a sequence with high HSC can be constructed of sequence segments that are maximally similar to different members of the set of host sequences at different positions.

[122] The above measure of hostness is likely to be more immunologically relevant than the more commonly used global identity measure of equation 4:

Equation 4 $$globalID = 100 \cdot \max_{h \in HS} \frac{1}{L} \sum_{i=1}^{L} \delta_{aa_i^s, aa_i^h}$$

Equation 4 disregards the extent of contiguous sequence identity, which is particularly relevant for capturing the molecular behavior of the immune system.

[123] Additional scoring functions similar to equations 3 are also possible. For example, as will be appreciated by those skilled in the art, there is some uncertainty regarding the hostness of a string wherein IDmax = w-1, w-2, etc. In one alternative embodiment, sequence similarity is compared instead of identity, using any of a variety of amino acid substitution matrices (e.g. PAM, BLOSUM62, etc.), providing a host string similarity (HSS) score as in equation 5:

Equation 5 $$HSS(s) = 100 \cdot \frac{1}{L-w+1} \sum_{i=1}^{L-w+1} \max_{h \in HS} (e^{\sum_{j=i}^{i+w-1} (S_{aa_j^s, aa_j^h} - S_{aa_j^s, aa_j^s})})$$

where S is a substitution score comparing any two amino acids. In yet another alternative, sequence identities are weighted according to the extent of identity, as in equation 6:

Equation 6 $$HSC(s) = 100 \cdot \frac{1}{(L-w+1)} \sum_{i=1}^{L-w+1} f(\max_{h \in HS} (\sum_{j=i}^{i+w-1} \delta_{aa_j^s, aa_j^h}))$$

where f is a continuous or noncontinuous function dependent on IDmax. For example, perfect matches can be weighted greater than near perfect matches (e.g. f(w)=1, f(w-1)=.5, etc.), and poor matches can be discarded (e.g. f(w-3)=f(w-2) = 0).

String window size

[124] The fundamental binding units of class I and class II MHC proteins are both 9 amino acids. In a preferred embodiment, the window size w used to create and score parent sequences is 9. However, it is also known that additional peptide flanking residues (PFRs) can influence T-cell recognition (via the TCR) of class II MHC-peptide complexes (see for example Arnold et al., 2002, *J Immunology* 169(2): 739-49), with the residues at positions P-1 (one position before the 1st MHC binding position) and P11 being most influential.

Because these effects might influence immune tolerance, a desirable goal of the invention, larger window sizes (e.g. 12) can be used. It should be noted however, that sequences optimized with similar window sizes are highly correlated.

Optimization of HSC

[125] Although a definition of string scoring systems is useful, an efficient process for discovering sequences with high HSC is also desirable. It is therefore a further aspect of the invention to provide methods for dynamic optimization of HSC given the described scoring systems.

[126] Desirable features of an optimization method include but are not limited to the following: 1) the output sequences are optimal or near-optimal (subject to design constraints) in their host string content; 2) structural constraints can be used to modulate the nature of the optimized sequences; and 3) multiple near-optimal solutions can be generated. Additionally, in some preferred embodiments, host string content may be maximized using a minimal number of substitutions.

[127] In a preferred embodiment, an iterative algorithm for optimization of HSC works as follows. 1) a parent sequence and set of host sequences are defined; 2) mutational constraints are defined at functionally or structurally important positions, referred to herein as masking - in a preferred embodiment, for antibody applications, positions within or structurally proximal to CDR residue (as defined by herein, or alternatively as defined by Kabat or Chothia) and/or interface are masked, locked, or fixed so that mutations are not possible (in some embodiments this constraint can be relaxed if the potential mutation is a conservative substitution of the parent amino acid). In a preferred embodiment, positions within 5 angstroms of a CDR residue or interface are masked. In other preferred embodiments, positions within 6.5 angstroms of a CDR residue or interface are masked; 3) host sequence segments (up to a defined length: lengths from 1-6 are typical) are collected from the alignment and stored for each position: segments that violate the mutational constraints are not collected; 4) each segment is analyzed for its potential impact on HSC, in the context of the current parent sequence, defined as String Impact (SI) in equation 7:

Equation 7 $$SI(x_m(z) \rightarrow y_m(z)) = HSC(s(y_m)) - HSC(parent)$$

where $y_m(z)$ is a host segment of length z replacing segment x at position m, and $s(y_m)$ and parent are versions of the parent sequence that include these segments (the parent sequence contains $x_m(z)$). 5) a single string is randomly selected from all stored host strings; the probability of selection is biased and proportional to the impact on HSC and inversely

proportional to the number of mutations relative to the current parent sequence, as in Equation 8:

$$P \propto \frac{SI(x_m(z) \rightarrow y_m(z))}{\sum_{i=m}^{m+z-1} \delta_{aa_i^{parent}, aa_i^z(y_m)}}$$

Equation 8

This selected string is substituted into the current parent sequence for its corresponding parent amino acids on an amino acid string by amino acid string basis. This kind of selection bias tends to optimize host string content with minimal perturbation of the original sequence. 6) steps 4 and 5 are repeated until no further optimization is possible (no segment substitutions have a favorable impact on host string content).

[128] Such an algorithm is inherently non-deterministic, so independent runs of the algorithm will tend to generate different solutions (this is a favorable feature). In a preferred embodiment, such an algorithm is applied numerous times to generate a diverse array of unique solutions. These solutions can be further clustered such that representative sequences can be prioritized for further analysis. For example, in one embodiment, the solution sequences are clustered into groups of similar sequences according to mutational distance, using a nearest neighbor single linkage hierarchical clustering algorithm to assign sequences to related groups based on similarity scores. Clustering algorithms may be useful for classifying sequences into representative groups. Representative groups may be defined, for example, by similarity. Measures of similarity include, but are not limited to sequence similarity and energetic similarity. Thus the output sequences from computational screening may be clustered around local minima, referred to herein as clustered sets of sequences. Sets of sequences that are close in sequence space may be distinguished from other sets. In one embodiment, diversity across clustered sets of sequences may be sampled by experimentally testing only a subset of sequences within each clustered set. For example, all or most of the clustered sets could be broadly sampled by including the lowest energy sequence from each clustered set of sequences to be experimentally tested. Because the sequence space of solutions with optimized HSC can be large, additional methods can be applied to ensure that a broad set of sequences is created. In a preferred embodiment, individual framework sequences generated by the procedure are clustered separately to generate a list of nonredundant basis framework regions (FRs) with high HSC. These basis FRs are then computationally assembled in all combinations along with the CDRs to generate a secondary list of solution sequences (which will usually have some overlap with the primary set). Alternatively, the basis FRs may be combined into an

experimental library, for example a combinatorial library.

Framework Diversity

[129] Application of this algorithm will generate variant protein solutions for which HSC is higher than the original parent sequence. It will also frequently generated solutions in which substituted strings are derived from different members of the alignment. The variant sequences derived using the present invention generally have unique properties relative to sequences generated using other methodologies. For example, in the context of an antibody, the protein variants of the invention frequently derive their host string content from a combination of different host germline sequences. This may be true even within a single FR. Quantification of these properties is useful for defining the nature of sequences derived using the present invention. A clear distinction emerges from a comparison of exact string content (meaning a perfect match over window w) in any single germline sequence versus exact w-mer string content within the set of all germline sequences (content of strings for which IDmax = w). Single germline exact string content (SGESC) of a variant sequence v may thus be defined as:

Equation 9 $$SGESC(v) = 100 \cdot \frac{1}{L-w+1} \cdot \max_{h \in HS} \sum_{i=1}^{L-w+1} \delta_{aa^{v}_{i\_i+w-1}, aa^{h}_{i\_i+w-1}}$$

[130] This quantity provides the extent to which a string-optimized sequence has string identity with the closest single germline sequence. Using this definition, it is also possible to assess the extent to which the high host string content of a given variant sequence v is derived from a single germline as opposed to multiple germline sequences. Framework region homogeneity (FRH) is defined as follows:

Equation 10 $$FRH(v) = \frac{SGESC(v)}{ESC(v)} = \frac{\max_{h \in HS} \sum_{i=1}^{L-w+1} \delta_{aa^{v}_{i\_i+w-1}, aa^{h}_{i\_i+w-1}}}{\sum_{i=1}^{L-w+1} \max_{h \in HS} \delta_{aa^{v}_{i\_i+w-1}, aa^{h}_{i\_i+w-1}}}$$

[131] In other words, if a variant sequence's exact string content is derived solely from a single germline sequence, the FRH would be close to 1.0. It should be noted that a similar or identical quantity can be defined for non-antibody proteins. Alternatively, as is the case with many of the variant sequences created by the present invention, FRH values can be significantly less than 1, with values ranging from 0.4 to 1.0, indicating, as expected, that sequences with high exact string content can be discovered with contributions from multiple

germline subfamilies and sequences. As described more fully in Example 5 below, variant sequences generated using the present invention have high HSC values yet many have low FRH values, indicating their HSC is derived from multiple germline frameworks.

Additional Scoring

[132] The above methods of scoring use the information present in an aligned set of host sequences as a metric of immunogenicity to maximize the content of host linear sequence strings in a parent sequence. In addition to such scoring functions, other scoring functions and methods may be employed. Such additional scoring functions may be aimed at the same goal as the aforementioned linear string scoring function, namely immunogenicity reduction of the parent protein. Alternatively, such additional scoring functions may be used to achieve other goals, for example optimization of protein stability, solubility, expression, pharmacokinetics, and/or aspects of protein function such as affinity of the parent protein for a target ligand, specificity, effector function, and/or enzymatic activity. For example an additional scoring function may be employed to enhance the affinity of an antibody variable domain for its target antigen. Such additional scoring functions may be employed statically or dynamically for the generation of optimized protein variants. A number of embodiments are described below as preferred additional scoring functions that may be used with the aforementioned linear string scoring method of the present invention. However, these are not meant to constrain the invention to these embodiments, and it should be clear that any method of scoring the fitness of an amino acid modification in a parent protein may be coupled with the novel linear string scoring method of the present invention so that optimal protein variants may be designed.

[133] In a preferred embodiment, substitutions are scored based on their structural compatibility with the structure of the parent protein. Such methods of scoring may require the structural coordinates that describe the three-dimensional structure of the protein, for example as obtained by X-ray crystallographic and nuclear magnetic resonance (NMR) techniques. Suitable proteins structures may also be obtained from structural models, which may be generated by methods that are known in the art of structural biology, including but not limited to *de novo* and homology modeling. Structure-based scoring functions may include any number of potentials that describe or approximate physical or chemical energy terms, including but not limited to a van der Waals potential, a hydrogen bond potential, an atomic solvation potential or other solvation models, a secondary structure propensity potential, an electrostatic potential, a torsional potential, an entropy potential, and/or additional energy terms. In other preferred embodiments, scoring methods may also be

derived from sequence information, including but not limited to knowledge-based potentials derived from protein sequence and/or structure statistics, threading potentials, reference energies, pseudo energies, homology-based energies, and sequence biases derived from sequence alignments. In alternately preferred embodiments, both structural and sequence-based potentials are used to generate one or more scoring functions that may be coupled with the linear string scoring method of the present invention.

[134] In a most preferred embodiment, a scoring method is used wherein the structural and functional integrity of substitutions are evaluated using a sequence and structure-based scoring function described in USSN 60/528,229, filed December 8, 2003, entitled Protein Engineering with Analogous Contact Environments; and USSN 60/602,566, filed August 17, 2004, entitled Protein Engineering with Analogous Contact Environments. This method combines sequence alignment information and structural information to predict the structural compatibility of one or more substitutions with a protein structure template. Nearest neighbor structure-based scores generated by this method include Structural Consensus and Structural Precedence as provided in the Examples. This method is particularly well suited for application to evaluating the structural fitness of immunoglobulins due to their substantial sequence and structural homology.

[135] In a preferred embodiment, substitutions are scored using a scoring function or computational design program that is substantially similar to Protein Design Automation® (PDA®) technology, as is described in US 6,188,965; US 6,269,312; US 6,403,312; US 6,708,120; US 6,804,611; US 6,792,356; USSN 09/782,004; USSN 09/812,034; USSN 09/927,790; USSN 10/218,102; USSN 10/101,499; USSN 10/218,102; USSN 10/666,311; USSN 10/665,307; USSN 10/888,748; PCT WO 98/07254; PCT WO 99/24229; PCT WO 01/40091; and PCT WO 02/25588. In another preferred embodiment, a computational design method substantially similar to Sequence Prediction Algorithm™ (SPA™) technology is used, as is described in (Raha *et al.*, 2000, *Protein Sci.* 9: 1106-1119), USSN 09/877,695, and USSN 10/071,859. In another preferred embodiment, the computational methods described in USSN 10/339,788, are used.

[136] In another preferred embodiment, optimized sequences are also assessed for surface similarity with host antibodies. Ensuring similarity may be important for reducing the probability of introducing novel 3D epitopes, which are potentially recognized by B-cell receptors. In a preferred embodiment, surface similarity at position i is quantified as follows:

Equation 11 $$surfscore(i) = \max_{k \in HS} e^{f_i^{cap} \cdot ((\sum_{j=1}^{L} proximity(i,j) * S(aa_j^s, aa_j^k)) - (\sum_{j=1}^{L} proximity(i,j) * S(aa_j^s, aa_j^s)))/T}$$

where f_i^{exp} is the fraction accessibility of position i to solvent, proximity(i,j) is the spatial proximity of positions i and j in the three-dimensional structure of the protein, S is a measure of amino acid similarity, and T is a temperature factor used to tune the stringency of the similarity comparison. It will be appreciated from the equation that if sequences are identical in the region of position i, a surfscore of 1.0 will be approached. Alternatively, a score of 1.0 can also be achieved if a position is completely buried (i.e. $f_i^{exp}=0$), since the position would not be accessible to B-cell receptors. Lower scores represent surface positions for which there are significant differences between the variant sequence and the most similar host sequence. In a preferred embodiment, the proximity between two positions is inversely related to their distance (e.g. a Gaussian or exponential function of the distance), and the proximity of a position to itself is 1.0. In a preferred embodiment, the decay of the proximity function is tuned such that patches of positions correspond to the size of a typical antibody epitope.

[137] Other surface properties may also be desirable. For example, optimized variant sequences may be assessed for the exposure of nonpolar amino acids, which is generally expected to decrease solubility. In such cases, variant sequences with lower nonpolar exposure can be prioritized over alternatives. Surface electrostatic properties may also be assessed for variant sequences. In a preferred embodiment, surface properties are assessed for multiple variants with optimized HSC, in order to limit the set of variants that will be experimentally screened.

[138] In another embodiment, substitution matrices or other knowledge-based scoring methods are used to identify alternate sequences that are likely to retain the structure and function of the protein. Such scoring methods can be used to quantify how conservative a given substitution or set of substitutions is. In most cases, conservative mutations do not significantly disrupt the structure and function of proteins (see for example, Bowie *et. al.*, 1990, *Science* 247: 1306-1310, Bowie & Sauer, 1989, *Proc. Nat. Acad. Sci. USA* 86: 2152-2156, and Reidhaar-Olson & Sauer, 1990, *Proteins* 7: 306-316). However, non-conservative mutations can destabilize protein structure and reduce activity (see for example, Lim *et al.*, 1992, *Biochem.* 31: 4324-4333). Substitution matrices including but not limited to BLOSUM62 provide a quantitative measure of the compatibility between a sequence and a target structure, which can be used to predict non-disruptive substitution mutations (Topham *et al.*, 1997, *Prot. Eng.* 10: 7-21). The use of substitution matrices to design peptides with improved properties has been disclosed (Adenot *et al.*, 1999, *J. Mol. Graph. Model.* 17: 292-309). Substitution matrices include, but are not limited to, the BLOSUM matrices (Henikoff & Henikoff, 1992, *Proc. Nat. Acad. Sci. USA* 89: 10917, the PAM matrices, the Dayhoff matrix,

and the like. For a review of substitution matrices, see for example Henikoff, 1996, *Curr. Opin. Struct. Biol.* 6: 353-360. It is also possible to construct a substitution matrix based on an alignment of a given protein of interest and its homologs; see for example Henikoff & Henikoff, 1996, *Comput. Appl. Biosci.* 12: 135-143.

[139] In a preferred embodiment, other methods for scoring immunogenicity may additionally be used. Most preferably, immunogenicity may be scored using a function that considers peptide binding to one or more MHC molecules. For example, substitutions would be scored such that there are no or a minimal number of immune epitopes that are predicted to bind, with high affinity, to any prevalent MHC alleles. These methods of scoring may be useful, for example, for designing substitutions in VH CDR3, for which scoring using human germline strings may be less straightforward. Several methods of identifying MHC-binding epitopes in protein sequences are known in the art and may be used to score epitopes in an antibody. See for example WO 98/52976; WO 02/079232; WO 00/3317; USSN 09/903,378; USSN 10/039,170; USSN 60/222,697; USSN 10/339788; PCT WO 01/21823; and PCT WO 02/00165; Mallios, 1999, *Bioinformatics* 15: 432-439; Mallios, 2001, *Bioinformatics* 17: 942-948; Sturniolo *et al.*, 1999, *Nature Biotech.* 17: 555-561; WO 98/59244; WO 02/069232; WO 02/77187; Marshall *et al.*, 1995, *J. Immunol.* 154: 5927-5933; and Hammer *et al.*, 1994, *J. Exp. Med.* 180: 2353-2358. Sequence-based information can be used to determine a binding score for a given peptide – MHC interaction (see for example Mallios, 1999, *Bioinformatics* 15: 432-439; Mallios, 2001, *Bioinformatics* 17: p942-948; Sturniolo *et. al.*, 1999, *Nature Biotech.* 17: 555-561). It is possible to use structure-based methods in which a given peptide is computationally placed in the peptide-binding groove of a given MHC molecule and the interaction energy is determined (for example, see WO 98/59244 and WO 02/069232). Such methods may be referred to as "threading" methods. Alternatively, purely experimental methods can be used; for example a set of overlapping peptides derived from the protein of interest can be experimentally tested for the ability to induce T-cell activation and/or other aspects of an immune response. (see for example WO 02/77187). In a preferred embodiment, MHC-binding propensity scores are calculated for each 9-residue frame along the protein sequence using a matrix method (see Sturniolo *et. al., supra*; Marshall *et. al.*, 1995, *J. Immunol.* 154: 5927-5933, and Hammer *et. al.*, 1994, *J. Exp. Med.* 180: 2353-2358). It is also possible to consider scores for only a subset of these residues, or to consider also the identities of the peptide residues before and after the 9-residue frame of interest. The matrix comprises binding scores for specific amino acids interacting with the peptide binding pockets in different human class II MHC molecule. In the most preferred embodiment, the scores in the matrix are obtained from experimental peptide binding

studies. In an alternate preferred embodiment, scores for a given amino acid binding to a given pocket are extrapolated from experimentally characterized alleles to additional alleles with identical or similar residues lining that pocket. Matrices that are produced by extrapolation are referred to as "virtual matrices".

[140] In alternate embodiments, additional scoring functions are employed that predict reactive sites within a protein, such as deamidation sites, glycosylation sites, oxidation sites, protealytic cleavage sites, and the like.

[141] It will be appreciated by one of skill in the art that the use of combinations of any of the aforementioned scoring functions and/or other scoring functions is contemplated. In one embodiment, this could be accomplished by evaluating the outputs of the results from separate calculations. Alternatively, scoring functions may be combined into one scoring term. This latter strategy enables different scoring terms to be weighted separately, thus providing more control over the relative contributions of the scoring terms, and a greater capacity to tune the scoring function for a desired engineering strategy.

Additional Optimization of Sequences

[142] In a preferred embodiment, after optimized protein variants have been engineered using the aforementioned scoring functions, additional optimization of protein variants may be carried out. In this way, an optimized protein variant can be thought of as primary variant or template for further optimization, and variants of this primary variant can be thought of as secondary variants. Because variant sequences of the invention are preferably derived from a HSC-increasing procedure in which substitution of structurally important positions is disallowed or discouraged (for example masking), it is likely that additional optimization of HSC is possible if those positions are allowed to vary in a secondary analysis. Optimization of other properties is possible, including but are not limited to protein affinity, expression, specificity, solubility, activity, and effector function. Thus the variant sequences derived in the primary analysis can represent variants for further optimization. In a preferred embodiment, the secondary analysis comprises the steps of 1) string analysis of the template sequence to identify secondary amino acid diversity that will have neutral, positive, or minimal impact on HSC; 2) experimental production of secondary variant sequences using the diversity derived in step 1; and 3) experimental screening of secondary variant sequences.

[143] For these purposes, the string impact (SI) of a single substitution at position m from amino acid x to amino acid y can be quantified as in Equation 7 (where segment length z = 1). As will be appreciated, the maximum possible string increase (for a single substitution at

an internal position) is w and the maximum possible decrease is w.

[144] In a most preferred embodiment, substitutions in a primary variant are chosen as those substitutions that will result in zero or positive string impact, referred to herein as string neutral and string positive substitutions respectively. In other embodiments, substitutions that result in negative string impact, i.e. string negative substitutions, may also be considered for engineering secondary variants. Secondary variants then may be constructed, expressed, and tested experimentally. Secondary substitutions that show favorable properties with respect to antigen affinity, effector function, stability, solubility, expression, and the like, may be combined in subsequent variants to generate a more optimized therapeutic candidate.

Optimization of Non-Xenogeneic Proteins

[145] It can be appreciated that the optimization described above is not restricted to immunogenicity reduction of xenogeneic proteins. Evaluation of potential substitutions for string impact provides an excellent strategy for generating substitution diversity for engineering protein variants with optimized properties. A clear advantage of this approach is that it generates protein variants with minimal immunogenicity risk, the importance of which has been discussed extensively and is a primary goal of the present invention. An additional advantage of this approach is that because the sequences being used to evaluate string impact are typically derived from a set of naturally evolved host sequences, variants designed are effectively enriched for stability, solubility, and other favorable properties. The utility of this capability lies in the fact that there are innumerable amino acid modifications that are detrimental or deleterious to proteins. By screening a quality set of variant diversity, the chances are increased that a protein variant of the desired property will be obtained. The capacity of the string impact approach to generate a quality set of variant diversity derives from the greater tolerance to mutation of positions which sample greater diversity, and the greater propensity of amino acids in a set of naturally evolved sequences to be compatible with a homologous protein's structure, stability, solubility, function, and the like.

[146] This string impact approach to variant design may be applied not only to the generation of secondary variants as described above, but may also be used to engineer amino acid modifications in proteins that are presumably already minimally immunogenic. This may include, for example, natural host proteins. Alternatively, and in a preferred embodiment, the string impact strategy may be applied to engineer modifications in an antibody variable region (VH or VL) that is humanized (Clark, 2000, *Immunol Today* 21:397-402), or "fully human" as obtained for example using transgenic mice (Bruggemann *et al.*,

1997, *Curr Opin Biotechnol* 8:455-458) or human antibody libraries coupled with selection methods (Griffiths *et al.*, 1998, *Curr Opin Biotechnol* 9:102-108). As with optimization of primary variant sequences described above, the string impact analysis described here can be used to identify secondary diversity that will have neutral, positive, or minimal impact on HSC, as well as potentially other favorable properties. Such diversity can then be used to screen for optimized versions of these sequences without increasing the risk of immunogenicity.

Experimental Production, Screening, and Testing

[147] Methods for production and screening of protein variants are well known in the art. General methods for antibody molecular biology, expression, purification, and screening are described in Antibody Engineering, edited by Duebel & Kontermann, Springer-Verlag, Heidelberg, 2001; and Hayhurst & Georgiou, 2001, *Curr Opin Chem Biol* 5:683-689; Maynard & Georgiou, 2000, *Annu Rev Biomed Eng* 2:339-76. Also see the methods described in USSN 10/339788, filed on March 3, 2003, USSN 10/672,280, filed September 29, 2003, and USSN 10/822,231, filed March 26, 2004.

[148] In one embodiment of the present invention, the library sequences are used to create nucleic acids that encode the member sequences, and that may then be cloned into host cells, expressed and assayed, if desired. These practices are carried out using well-known procedures, and a variety of methods that may find use in the present invention are described in Molecular Cloning - A Laboratory Manual, 3rd Ed. (Maniatis, Cold Spring Harbor Laboratory Press, New York, 2001), and Current Protocols in Molecular Biology (John Wiley & Sons). The nucleic acids that encode the protein variants of the present invention may be incorporated into an expression vector in order to express the protein. Expression vectors typically comprise a protein operably linked, that is placed in a functional relationship, with control or regulatory sequences, selectable markers, any fusion partners, and/or additional elements. The protein variants of the present invention may be produced by culturing a host cell transformed with nucleic acid, preferably an expression vector, containing nucleic acid encoding the protein variants, under the appropriate conditions to induce or cause expression of the protein. A wide variety of appropriate host cells may be used, including but not limited to mammalian cells, bacteria, insect cells, and yeast. For example, a variety of cell lines that may find use in the present invention are described in the ATCC cell line catalog, available from the American Type Culture Collection. The methods of introducing exogenous nucleic acid into host cells are well known in the art, and will vary with the host cell used.

[149] In a preferred embodiment, protein variants are purified or isolated after expression. Proteins may be isolated or purified in a variety of ways known to those skilled in the art. Standard purification methods include chromatographic techniques, electrophoretic, immunological, precipitation, dialysis, filtration, concentration, and chromatofocusing techniques. As is well known in the art, a variety of natural proteins bind antibodies, for example bacterial proteins A, G, and L, and these proteins may find use in the present invention for purification. Purification can often be enabled by a particular fusion partner. For example, proteins may be purified using glutathione resin if a GST fusion is employed, Ni^{+2} affinity chromatography if a His-tag is employed, or immobilized anti-flag antibody if a flag-tag is used. For general guidance in suitable purification techniques, see Protein Purification: Principles and Practice, 3rd Ed., Scopes, Springer-Verlag, NY, 1994.

[150] Protein variants may be screened using a variety of methods, including but not limited to those that use *in vitro* assays, *in vivo* and cell-based assays, and selection technologies. Automation and high-throughput screening technologies may be utilized in the screening procedures. Screening may employ the use of a fusion partner or label, for example an immune label, isotopic label, or small molecule label such as a fluorescent or colorimetric dye.

[151] In a preferred embodiment, the functional and/or biophysical properties of protein variants are screened in an *in vitro* assay. In a preferred embodiment, the protein is screened for functionality, for example its ability to catalyze a reaction or its binding affinity to its target. Binding assays can be carried out using a variety of methods known in the art, including but not limited to FRET (Fluorescence Resonance Energy Transfer) and BRET (Bioluminescence Resonance Energy Transfer) -based assays, AlphaScreen™ (Amplified Luminescent Proximity Homogeneous Assay), Scintillation Proximity Assay, ELISA (Enzyme-Linked Immunosorbent Assay), SPR (Surface Plasmon Resonance, also known as BIACORE®), isothermal titration calorimetry, differential scanning calorimetry, gel electrophoresis, and chromatography including gel filtration. These and other methods may take advantage of some fusion partner or label. Assays may employ a variety of detection methods including but not limited to chromogenic, fluorescent, luminescent, or isotopic labels. The biophysical properties of proteins, for example stability and solubility, may be screened using a variety of methods known in the art. Protein stability may be determined by measuring the thermodynamic equilibrium between folded and unfolded states. For example, protein variants of the present invention may be unfolded using chemical denaturant, heat, or pH, and this transition may be monitored using methods including but not limited to circular dichroism spectroscopy, fluorescence spectroscopy, absorbance

spectroscopy, NMR spectroscopy, calorimetry, and proteolysis. As will be appreciated by those skilled in the art, the kinetic parameters of the folding and unfolding transitions may also be monitored using these and other techniques. The solubility and overall structural integrity of a protein variant may be quantitatively or qualitatively determined using a wide range of methods that are known in the art. Methods which may find use in the present invention for characterizing the biophysical properties of protein variants include gel electrophoresis, chromatography such as size exclusion chromatography and reversed-phase high performance liquid chromatography, mass spectrometry, ultraviolet absorbance spectroscopy, fluorescence spectroscopy, circular dichroism spectroscopy, isothermal titration calorimetry, differential scanning calorimetry, analytical ultra-centrifugation, dynamic light scattering, proteolysis, and cross-linking, turbidity measurement, filter retardation assays, immunological assays, fluorescent dye binding assays, protein-staining assays, microscopy, and detection of aggregates via ELISA or other binding assay. Structural analysis employing X-ray crystallographic techniques and NMR spectroscopy may also find use.

[152] In a preferred embodiment, protein variants are screened using one or more cell-based or *in vivo* assays. For such assays, purified or unpurified proteins are typically added exogenously such that cells are exposed to individual variants or pools of variants belonging to a library. These assays are typically, but not always, based on the function of the protein; that is, the ability of the protein to bind to its target and mediate some biochemical event, for example effector function, ligand/receptor binding inhibition, apoptosis, and the like. Such assays often involve monitoring the response of cells to the protein, for example cell survival, cell death, change in cellular morphology, or transcriptional activation such as cellular expression of a natural gene or reporter gene. For example, such assays may measure the ability of antibody variants to elicit ADCC, ADCP, or CDC. For some assays additional cells or components, that is in addition to the target cells, may need to be added, for example serum complement, or effector cells such as peripheral blood monocytes (PBMCs), NK cells, macrophages, and the like. Such additional cells may be from any organism, preferably humans, mice, rat, rabbit, and monkey. Proteins may cause apoptosis of certain cell lines expressing the target, or they may mediate attack on target cells by immune cells which have been added to the assay. Methods for monitoring cell death or viability are known in the art, and include the use of dyes, immunochemical, cytochemical, and radioactive reagents. For example, caspase staining assays may enable apoptosis to be measured, and uptake or release of radioactive substrates or fluorescent dyes such as alamar blue may enable cell growth or activation to be monitored. In a preferred embodiment, the DELFIA®

EuTDA-based cytotoxicity assay (Perkin Elmer, MA) is used. Alternatively, dead or damaged target cells may be monitored by measuring the release of one or more natural intracellular proteins, for example lactate dehydrogenase. Transcriptional activation may also serve as a method for assaying function in cell-based assays. In this case, response may be monitored by assaying for natural genes or proteins which may be upregulated, for example the release of certain interleukins may be measured, or alternatively readout may be via a reporter construct. Cell-based assays may also involve the measure of morphological changes of cells as a response to the presence of a protein. Cell types for such assays may be prokaryotic or eukaryotic, and a variety of cell lines that are known in the art may be employed. Alternatively, cell-based screens are performed using cells that have been transformed or transfected with nucleic acids encoding the variant proteins. That is, protein variants are not added exogenously to the cells. For example, in one embodiment, the cell-based screen utilizes cell surface display. A fusion partner can be employed that enables display of variants on the surface of cells (Witrrup, 2001, *Curr Opin Biotechnol*, 12:395-399).

[153] As is known in the art, a subset of screening methods are those that select for favorable members of a library. The methods are herein referred to as "selection methods", and these methods find use in the present invention for screening protein variants. When protein libraries are screened using a selection method, only those members of a library that are favorable, that is which meet some selection criteria, are propagated, isolated, and/or observed. As will be appreciated, because only the most fit variants are observed, such methods enable the screening of libraries that are larger than those screenable by methods that assay the fitness of library members individually. Selection is enabled by any method, technique, or fusion partner that links, covalently or noncovalently, the phenotype of a protein with its genotype, i.e., the function of a protein with the nucleic acid that encodes it. For example the use of phage display as a selection method is enabled by the fusion of library members to the gene III protein. In this way, selection or isolation of protein variants that meet some criteria, for example binding affinity to the protein's target, also selects for or isolates the nucleic acid that encodes it. Once isolated, the gene or genes encoding variants may then be amplified. This process of isolation and amplification, referred to as panning, may be repeated, allowing favorable protein variants in the library to be enriched. Nucleic acid sequencing of the attached nucleic acid ultimately allows for gene identification.

[154] A variety of selection methods are known in the art that may find use in the present invention for screening protein libraries. These include but are not limited to phage display (Phage display of peptides and proteins: a laboratory manual, Kay *et al.*, 1996, Academic

Press, San Diego, CA, 1996; Lowman *et al.*, 1991, *Biochemistry* 30:10832-10838; Smith, 1985, *Science* 228:1315-1317) and its derivatives such as selective phage infection (Malmborg *et al.*, 1997, J Mol Biol 273:544-551), selectively infective phage (Krebber *et al.*, 1997, *J Mol Biol* 268:619-630), and delayed infectivity panning (Benhar *et al.*, 2000, *J Mol Biol* 301:893-904), cell surface display (Witrrup, 2001, *Curr Opin Biotechnol*, 12:395-399) such as display on bacteria (Georgiou *et al.*, 1997, *Nat Biotechnol* 15:29-34; Georgiou *et al.*, 1993, *Trends Biotechnol* 11:6-10; Lee *et al.*, 2000, *Nat Biotechnol* 18:645-648; Jun *et al.*, 1998, *Nat Biotechnol* 16:576-80), yeast (Boder & Wittrup, 2000, *Methods Enzymol* 328:430-44; Boder & Wittrup, 1997, *Nat Biotechnol* 15:553-557), and mammalian cells (Whitehorn *et al.*, 1995, *Bio/technology* 13:1215-1219), as well as *in vitro* display technologies (Amstutz *et al.*, 2001, *Curr Opin Biotechnol* 12:400-405) such as polysome display (Mattheakis *et al.*, 1994, *Proc Natl Acad Sci USA* 91:9022-9026), ribosome display (Hanes *et al.*, 1997, *Proc Natl Acad Sci USA* 94:4937-4942), mRNA display (Roberts & Szostak, 1997, *Proc Natl Acad Sci USA 94*:12297-12302; Nemoto *et al.*, 1997, *FEBS Lett* 414:405-408), and ribosome-inactivation display system (Zhou *et al.*, 2002, *J Am Chem Soc* 124, 538-543).

[155] Other selection methods that may find use in the present invention include methods that do not rely on display, such as *in vivo* methods including but not limited to periplasmic expression and cytometric screening (Chen *et al.*, 2001, *Nat Biotechnol* 19:537-542), the protein fragment complementation assay (Johnsson & Varshavsky, 1994, *Proc Natl Acad Sci USA* 91:10340-10344; Pelletier *et al.*, 1998, *Proc Natl Acad Sci USA* 95:12141-12146), and the yeast two hybrid screen (Fields & Song, 1989, *Nature* 340:245-246) used in selection mode (Visintin *et al.*, 1999, *Proc Natl Acad Sci USA* 96:11723-11728). In an alternate embodiment, selection is enabled by a fusion partner that binds to a specific sequence on the expression vector, thus linking covalently or noncovalently the fusion partner and associated variant library member with the nucleic acid that encodes them. For example, USSN 09/642,574; USSN 10/080,376; USSN 09/792,630; USSN 10/023,208; USSN 09/792,626; USSN 10/082,671; USSN 09/953,351; USSN 10/097,100; USSN 60/366,658; PCT WO 00/22906; PCT WO 01/49058; PCT WO 02/04852; PCT WO 02/04853; PCT WO 02/08023; PCT WO 01/28702; and PCT WO 02/07466 describe such a fusion partner and technique that may find use in the present invention. In an alternative embodiment, *in vivo* selection can occur if expression of the protein imparts some growth, reproduction, or survival advantage to the cell.

[156] A subset of selection methods referred to as "directed evolution" methods are those that include the mating or breading of favorable sequences during selection, sometimes with the incorporation of new mutations. As will be appreciated by those skilled in the art,

directed evolution methods can facilitate identification of the most favorable sequences in a library, and can increase the diversity of sequences that are screened. A variety of directed evolution methods are known in the art that may find use in the present invention for screening protein variants, including but not limited to DNA shuffling (PCT WO 00/42561 A3; PCT WO 01/70947 A3), exon shuffling (US 6,365,377; Kolkman & Stemmer, 2001, *Nat Biotechnol* 19:423-428), family shuffling (Crameri *et al.*, 1998, *Nature* 391:288-291; US 6,376,246), RACHITT™ (Coco *et al.*, 2001, *Nat Biotechnol* 19:354- 359; PCT WO 02/06469), STEP and random priming of *in vitro* recombination (Zhao *et al.*, 1998, *Nat Biotechnol* 16:258-261; Shao *et al.*, 1998, *Nucleic Acids Res* 26:681-683), exonuclease mediated gene assembly (US 6,352,842; US 6,361,974), Gene Site Saturation Mutagenesis™ (US 6,358,709), Gene Reassembly™ (US 6,358,709), SCRATCHY (Lutz et al., 2001, *Proc Natl Acad Sci USA* 98:11248-11253), DNA fragmentation methods (Kikuchi *et al.*, *Gene* 236:159-167), single-stranded DNA shuffling (Kikuchi *et al.*, 2000, *Gene* 243:133-137), and AMEsystem™ directed evolution protein engineering technology (Applied Molecular Evolution) (US 5,824,514; US 5,817,483; US 5,814,476; US 5,763,192; US 5,723,323).

[157] In a preferred embodiment, the immunogenicity of the protein variants is determined experimentally to confirm that the variants do have reduced or eliminated immunogenicity relative to the parent protein. Several methods can be used for experimental confirmation of epitopes. In a preferred embodiment, *ex vivo* T-cell activation assays are used to experimentally quantitate immunogenicity. In this method, antigen presenting cells and naïve T cells from matched donors are challenged with a peptide or whole protein of interest one or more times. Then, T cell activation can be detected using a number of methods, for example by monitoring production of cytokines or measuring uptake of tritiated thymidine. In the most preferred embodiment, interferon gamma production is monitored using Elispot assays (Schmittel *et. al.,* 2000, *J. Immunol. Meth.*, 24: 17-24). If sera are available from patients who have raised an immune response to protein, it is possible to detect mature T cells that respond to specific epitopes. In a preferred embodiment, interferon gamma or IL-5 production by activated T-cells is monitored using Elispot assays, although it is also possible to use other indicators of T cell activation or proliferation such as tritiated thymidine incorporation or production of other cytokines. Other suitable T cell assays include those disclosed in Meidenbauer *et al.*, 2000, *Prostate* 43, 88-100; Schultes & Whiteside, 2003, *J. Immunol. Methods* 279, 1-15; and Stickler *et al.*, 200, *J. Immunotherapy*, 23, 654-660. In a preferred embodiment, the PBMC donors used for the above-described T cell activation assays will comprise class II MHC alleles that are common in patients requiring treatment for protein responsive disorders. For example, for most diseases and disorders, it is desirable

to test donors comprising all of the alleles that are prevalent in the population. However, for diseases or disorders that are linked with specific MHC alleles, it may be more appropriate to focus screening on alleles that confer susceptibility to protein responsive disorders. In a preferred embodiment, the MHC haplotype of PBMC donors or patients that raise an immune response to the wild type or protein variant are compared with the MHC haplotype of patients who do not raise a response. This data may be used to guide preclinical and clinical studies as well as aiding in identification of patients who will be especially likely to respond favorably or unfavorably to the protein therapeutic.

[158] In an alternate preferred embodiment, immunogenicity is measured in transgenic mouse systems. For example, mice expressing fully or partially human class II MHC molecules may be used. In an alternate embodiment, immunogenicity is tested by administering the protein variants to one or more animals, including rodents and primates, and monitoring for antibody formation. Nonhuman primates with defined MHC haplotypes may be especially useful, as the sequences and hence peptide binding specificities of the MHC molecules in nonhuman primates may be very similar to the sequences and peptide binding specificities of humans. Similarly, genetically engineered mouse models expressing human MHC peptide-binding domains may be used (see for example Sonderstrup *et al.*, 1999, *Immunol. Rev.* 172: 335-343; and Forsthuber *et al.*, 2001, J. *Immunol.* 167: 119-125).

[159] The biological properties of the proteins of the present invention may be characterized in cell, tissue, and whole organism experiments. As is known in the art, drugs are often tested in animals, including but not limited to mice, rats, rabbits, dogs, cats, pigs, and monkeys, in order to measure a drug's efficacy for treatment against a disease or disease model, or to measure a drug's pharmacokinetics, toxicity, and other properties. The animals may be referred to as disease models. Therapeutics are often tested in mice, including but not limited to nude mice, SCID mice, xenograft mice, and transgenic mice (including knockins and knockouts). Such experimentation may provide meaningful data for determination of the potential of the protein to be used as a therapeutic. Any organism, preferably mammals, may be used for testing. For example because of their genetic similarity to humans, monkeys can be suitable therapeutic models, and thus may be used to test the efficacy, toxicity, pharmacokinetics, or other property of the proteins of the present invention. Tests of the in humans are ultimately required for approval as drugs, and thus of course these experiments are contemplated. Thus the proteins of the present invention may be tested in humans to determine their therapeutic efficacy, toxicity, immunogenicity, pharmacokinetics, and/or other clinical properties.

[160] In one embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes two or more amino acid substitutions derived from two or more natural antibodies. In this embodiment, a first resultant variant string in the variant antibody is rendered most homologous to a first natural antibody, a second resultant variant string in the variant antibody is rendered most homologous to the corresponding string in an second natural antibody, the substitutions are not in a CDR, and at least one resultant string is not a consensus of homologous natural sequences. In a preferred embodiment, the variant strings in the variant antibody do not include CDR residues. In a further preferred embodiment, the first and second natural antibodies are from different subfamilies.

[161] In another embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes two or more amino acid substitutions derived from three or more natural antibodies. In this embodiment, a first resultant variant string in the variant antibody is rendered most homologous to a first natural antibody, a second resultant variant string in the variant antibody is rendered most homologous to the corresponding string in an second natural antibody, a third resultant variant string in the variant antibody is rendered most homologous to the corresponding string in an third natural antibody, the substitutions are not in a CDR, and at least one resultant string is not a consensus of homologous natural sequences. In an additional embodiment, the first, second and third natural antibodies are from different subfamilies. If a further additional embodiment, the variant antibody further comprises a fourth resultant variant string that is rendered most homologous to the corresponding string in a fourth natural antibody. If an additional embodiment, the variant antibody includes at least one substitution that is made at a position that is not surface exposed, the first, second and third natural antibodies are from different antibody groups, one of the substitutions is made at a position that is part of the VH/VL interface, and at least one amino acid substitution is not a back mutation.

[162] In other embodiments, the first, second and third natural antibodies are from different antibody groups.

[163] In other embodiments, the variant antibody includes at least one substitution that is not surface exposed.

[164] In other embodiments, at least one of the substitutions is made at a position that is part of the VH/VL interface.

[165] In other embodiments, the variant antibody includes at least one amino acid substitution is not a back mutation.

[166] In one embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes a variant VH antibody region with host string content (HSC) greater than about 75%, and a framework region homogeneity (FRH) less than about 60%, wherein the HSC and FRH are calculated with a window size of 9.

[167] In one embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes a variant VH antibody region with exact string content greater than about 20%; and a framework region homogeneity less than about 60%, wherein the HSC and FRH are calculated with a window size of 9.

[168] In one embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes a variant VL antibody region with exact string content greater than about 35%, and a framework region homogeneity less than about 60%, wherein HSC and FRH are calculated with a window size of 9.

[169] In one embodiment of the present invention, a variant antibody for a host as compared to a parent antibody includes a first set of one or more amino acid substitutions from a first natural antibody and a second set of one or more amino acid substitutions from a second natural antibody, wherein the identity of said substituted amino acids from said second antibody differ from the corresponding amino acids of said first natural antibody, the substitutions are not in a CDR, and at least one substitution is not a consensus of homologous natural sequences. In an additional embodiment, the variant antibody further includes a third set of one or more amino acids substitutions from a third natural antibody wherein the identity of the substituted amino acids of said third set differ from the identity the corresponding amino acids from said first and second sets of amino acid substitutions. In a further embodiment, the variant antibody includes a fourth set of one or more amino acids from a fourth natural antibody wherein the identity of the substituted amino acids of said fourth set differ from the identity the corresponding amino acids from said first, second and third sets of amino acid substitutions. In other embodiments, the variant antibody includes multiple sets of one or more amino acids from multiple natural antibodies, wherein the identity of the substituted amino acids of any set differ from the identity the corresponding amino acids from the other sets of amino acid substitutions.

EXAMPLES

[170] Examples are provided below to illustrate the present invention. These examples are not meant to constrain the present invention to any particular application or theory of operation.

[171] For reference to immunoglobulin variable regions, positions are numbered according to the Kabat numbering scheme. For reference to immunoglobulin constant regions, positions are numbered according to the EU index as in Kabat (Kabat *et al.*, 1991, *Sequences of Proteins of Immunological Interest*, 5th Ed., United States Public Health Service, National Institutes of Health, Bethesda).

Example 1. Immunogenicity Reduction of AC10

[172] To illustrate application of the method described in the present invention, and to validate its broad applicability to immunogenicity reduction of proteins, a xenogeneic antibody example is provided using as the parent sequence the anti-CD30 antibody AC10 (Bowen *et al.* Journal of Immunology, 1993, 151: 5896). A structural model of the mouse AC10 variable region was constructed using standard antibody modeling methods known in the art. Figures 3 and 4 show the sequences, host string content, and structures of the AC10 VL and VH domains (referred to as L0 AC10 VL and H0 AC10 VH respectively). A CDR graft of this antibody was constructed by placing the AC10 CDRs into the context of the frameworks of the most homologous host germlines, determined to be vlk_4-1 for VL and vh_1-3 for VH using the sequence alignment program BLAST. The sequences and string content of these CDR grafts are shown in Figures 5 and 6, along with structures of modeled AC10 highlighting the mutational differences between the CDR grafted AC10 variable chains and WT.

[173] AC10 variants with reduced immunogenicity were generated by applying a string optimization algorithm on the WT AC10 VL and VH sequences. This algorithm heuristically samples multiple amino acid mutations that exist in the diversity of the human VLκ and VH germline sequences, and calculates the host string content (HSC) of each sequence according to Equation 3 described above, using a window size w=9. In this set of calculations, residues in the CDRs and close to a CDR or to the VL/VH interface were masked, that is were not allowed to mutate. CDRs were defined as a slightly smaller set of residues than the CDRs defined by Chothia (Chothia & Lesk, 1987, *J. Mol. Biol.* 196: 901-917; Chothia *et al.*, 1989, *Nature* 342: 877-883; Al-Lazikani *et al.*, 1997, *J. Mol. Biol.* 273: 927-948). For the purposes of the present invention, VL CDRs are herein defined to include residues at positions 27-32 (CDR1), 50-56 (CDR2), and 91-97 (CDR3), wherein the numbering is according to Chothia. Because the VL CDRs as defined by Chothia and Kabat are identical, the numbering of these VL CDR positions is also according to Kabat. For the purposes of the present invention, VH CDRs are herein defined to include residues at positions 27-33 (CDR1), 52-56 (CDR2), and 95-102 (CDR3), wherein the numbering is

according to Chothia. These VH CDR positions correspond to Kabat positions 27-35 (CDR1), 52-56 (CDR2), and 95-102 (CDR3). Masked residues in these calculations were set at positions 1-4, 25-34, 36, 38, 43, 44, 46, 48-58, 60, 63-69, 71, 87, and 89-98 for VL, and 2, 4, 24, 26-35, 37, 39, 44, 45, 47, 50-58, 60, 61, 71, 73, 76, 78, 91, and 93-106 for VH, wherein the numbering is according to Kabat. Masking of potentially critical residues is a conservative approach to generating more host antibody variants, however it is but one embodiment of the present invention, and calculations wherein positions are not masked are also contemplated. This calculation was run for AC10 VL and VH in 100 separate interactions, generating a set of diverse AC10 variants with more host string content than WT. Figure 7 shows the clustered nonredundant set of output sequences from these calculations for the AC10 VL and VH region, referred to as AC10 VL HSC Calculation 1 and AC10 VH HSC Calculation 1 respecitvely. For each iteration (Iter), the HSC (Eqation 3), HSS (Equation 5), and number (Mut) and identity (shaded residues) of mutations from WT are presented. In addition to the HSC score, each sequence was evaluated for its structural and functional integrity using a nearest neighbor structure-based scoring method (USSN 60/528,229, filed December 8, 2003, entitled Protein Engineering with Analogous Contact Environments). Two measures of structural fitness, referred to as "Structural Consensus" and "Structural Precedence", are also provided in the Figure 7. Although the Analogous Contact Environments method is particularly well-suited for antibodies because of the wealth of sequence and structure information, any structure-based and/or sequence-based scoring method may be used to evaluate the structural and functional fitness of the variant sequences. The output sequences were clustered based on their mutational distance from the other sequences in the set, and these clusters are delineated by the horizontal black lines in the Figure. The "Cluster" column provides the quantitative mutational distance between each sequence and the rest of the sequence in its cluster; sequences with a lower cluster value are more representative of that particular sequence cluster.

[174] These calculations were used to generate a set of AC10 VL and VH variants. In some cases, further substitutions were made to output sequences, using string and structural scores, as well as visual inspection of the modeled AC10 structure, to evaluate fitness. Figures 8 – 13 present the sequences, host string content, and mapped mutational differences on the modeled AC10 structure for each of the AC10 VL and VH variants. Iteration 36 from AC10 VL HSC calculation 1 served as the precursor for L1 AC10 VL, iteration 37 served as the precursor for L2 AC10 VL, and iteration 3 served as the precursor for L3 AC10 VL. Iteration 15 from AC10 VH HSC calculation 1 served as the precursor for H1 AC10 HL, iteration 55 served as the precursor for H2 AC10 VH, and iteration 18 served

as the precursor for H3 AC10 VH.

[175] Tables 1 and 2 present the number of mutations from the parent sequence, structural fitness scores, and host string scores for the AC10 VL and VH variants as compared to the WT and CDR grafted AC10 sequences. In addition to the aforementioned structural and host string analysis, each sequence was analyzed for its global homology to the host germline. The maximum identity match to the germline for each string in the sequences was also determined, referred to as N_{ID}max. This represents the total number of strings in each sequence whose maximum identity to the corresponding strings in the host germline is the indicated value. For w = 9, Tables 1 and 2 list N_9max, N_8max, N_7max, and $N_{\leq 6}$max for each sequence. N_9max represents the number of strings in the sequence for which 9 of 9 residues match at least one string in the host germline, N_8max represents the number of strings for which 8 of 9 residues match at least one string in the host germline, N_7max represents the number of strings for which 7 of 9 residues match at least one string in the host germline, and $N_{\leq 6}$max represents the number of strings for which 6 or less residues of 9 residues match at least one string in the host germline. This last category (ID $\leq$6) could, for example, be regarded as the number of poorly scoring strings. In addition to the aforementioned structural and host string analysis, each sequence was analyzed for its global homology to the host germline; Tables 1 and 2 present the most homologous human germline sequence for each sequence (Closest Germline) and corresponding identity to that germline (ID to Closest Germline), determined using the sequence alignment program BLAST. Finally, the Framework region homogeneity (FRH) of each variant was evaluated for w=9, and is presented in Tables 1 and 2, providing the extent to which the host string content of each variant is derived from a single germline as opposed to multiple germline sequences.

Table 1. AC10 VL Variants

	WT	CDR Graft	L1	L2	L3
Mutations		18	15	16	9
Structural Consensus	0.57	0.57	0.59	0.64	0.56
Structural Precedence	0.68	0.57	0.66	0.67	0.58
Human String Content	0.78	0.88	0.86	0.86	0.85
Human String Similarity	0.15	0.57	0.48	0.46	0.41

	WT	CDR Graft	L1	L2	L3
Framework Region Homogeneity	0.60	0.97	0.73	0.81	0.52
N_9max	15	61	51	48	42
N_8max	27	11	13	15	21
N_7max	31	15	21	22	20
$N_{\leq 6}$max	34	20	22	22	24
Closest Germline	4-1	4-1	3-11	1-39	4-1
ID to Closest Germline	68 / 101 67%	86 / 101 85%	78 / 99 79%	80 / 99 81%	75 / 101 74%

Table 2. AC10 VH Variants

	WT	CDR Graft	H1	H2	H3
Mutations		26	16	23	20
Structural Consensus	0.49	0.48	0.48	0.50	0.47
Structural Precedence	0.63	0.67	0.63	0.59	0.59
Human String Content	0.69	0.87	0.81	0.81	0.80
Human String Similarity	0.07	0.68	0.41	0.39	0.38
Framework Region Homogeneity	0.60	0.86	0.65	0.47	0.55
N_9max	5	81	48	45	44
N_8max	31	13	30	32	28
N_7max	34	8	20	21	24
$N_{\leq 6}$max	49	17	21	21	23
Closest Germline	1-3	1-3	1-3	1-3	7-4-1
ID to Closest Germline	69 / 98 70%	93 / 98 95%	83 / 98 85%	72 / 98 73%	76 / 98 78%

[176] An important observation is that, whereas the CDR grafted antibodies are most homologous to a single human germline sequence (the "acceptor" sequence in humanization terminology), the present invention describes variants that are homologous to different host germline sequences in different regions of the sequence. This is evident from the significant differences in Framework region homogeneity (FRH) scores for the AC10 variants of the present invention and CDR grafted AC10 variants. Furthermore, whereas CDR grafted

AC10 VL and VH are most homologous to human germline subfamilies 4 (VL) and 1 (VH) respectively across their entire sequences, a number of the AC10 variants are most homologous to different subfamilies in different frameworks. Additionally, whereas the CDR grafted antibodies are most homologous to a single germline sequence that is also the most homologous sequence to the parent sequence, the present invention presents a set of antibodies for a given antibody that are most homologous to different human germline sequences, which need not be the most homologous germline sequence to WT. For example, Table 1 shows that CDR grafted AC10 VL is most homologous to 4-1, which is also the most homologous human germline to the WT AC10 parent. However L1, L2, and L3 are most homologous to three different human germlines – 3-11, 1-39, and 4-1 respectively. Thus the variants of the present invention explore a substantially greater amount of diversity than CDR grafted antibodies. One obvious advantage of this is that the method of the present invention provides a greater chance of success with respect to antigen affinity. The choice of an "acceptor" in humanization methods places a single bet; if the donor CDRs are in fact incompatible with the acceptor FRs, a set of backmutations that regain WT affinity may not exist. In contrast, the method of the present invention enables a greater diversity of sequence and structure space to be sampled in the immunogenicity reduction process, increasing the chances of obtaining a final less immunogenic version with WT affinity or better. An additional advantage of sampling greater sequence diversity is that some sequences may have more optimal properties than others, for example with regard to stability, solubility, and effector function. For example, as disclosed in USSN 60/614,944, and USSN 60/619,409, filed October 14, 2004, entitled "Immunoglobulin Variants Outside the Fc Region with Optimized Effector Function", the variable region of an antibody may impact effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP), and complement dependent cytotoxicity (CDC).

[177] The genes for the variable regions of AC10 WT (L0 and H0) and variants (L1, L2, L3, H1, H2, and H3) were constructed using recursive PCR, and subcloned into a the mammalian expression vector pcDNA3.1Zeo (Invitrogen) comprising the full length light kappa (CLκ) and heavy chain IgG1 constant regions. All sequences were sequenced to confirm the fidelity of the sequence. Plasmids containing heavy chain gene (VH-CH1-CH2-CH3) (wild-type or variants) were co-transfected with plasmid containing light chain gene (VL-CL□) in all combinations (L0/H0, L0/H1, L0/H2, L0/H3, L1/H0, L1/H1, L1/H2, L1/H3, L2/H0, L2/H1, L2/H2, L2/H3, L3/H0, L3/H1, L3/H2, L3/H3) into 293T cells. Here, for example, L2/H3 refers to the L2 AC10 VL paired with H3 AC10 VH. Media were harvested 5

days after transfection, and antibodies were purified from the supernatant using protein A affinity chromatography (Pierce, Catalog # 20334).

[178] WT and variant antibodies were experimentally tested for their capacity to bind CD30 antigen. Binding affinity to human CD30 by the AC10 WT and variant antibodies was measured using a quantitative and extremely sensitive method, AlphaScreen™ assay. The AlphaScreen™ assay is a bead-based non-radioactive luminescent proximity assay. Laser excitation of a donor bead excites oxygen, which if sufficiently close to the acceptor bead will generate a cascade of chemiluminescent events, ultimately leading to fluorescence emission at 520-620 nm. The AlphaScreen™ assay was applied as a competition assay for screening the antibodies. WT AC10 antibody was biotinylated by standard methods for attachment to streptavidin donor beads (Perkin Elmer). Commericial CD30 was conjugated to digoxigenin (DIG) (Roche Diagnostics) for attachment to anti-DIG acceptor beads (Perkin Elmer). In the absence of competing AC10 variants, WT antibody and CD30 interact and produce a signal at 520-620 nm. Addition of untagged AC10 variant competes with the WT AC10 / CD30 interaction, reducing fluorescence quantitatively to enable determination of relative binding affinities. Figures 14a and 14b show binding of WT (H0L0) and AC10 variant antibodies to CD30 using the AlphaScreen™ assay. The data were fit to a one site competition model using nonlinear regression, and these fits are represented by the curves in the figure. These fits provide the inhibitory concentration 50% (IC50) (i.e. the concentration required for 50% inhibition) for each antibody, thus enabling the relative binding affinities relative to WT to be determined. Table 3 provides the IC50's and Fold IC50's relative to WT for fits to these binding curves. The AC10 variants display an array of CD30 binding affinities, with a number of variants binding CD30 with affinity comparable to or better affinity than WT AC10.

[179] Antigen affinity of the AC10 variants was also measured using Surface Plasmon Resonance (SPR) (Biacore, Uppsala, Sweden). SPR allows for the measurement of direct binding rates and affinities of protein-protein interactions, and thus provides an excellent complementary binding assay to the AlphaScreen™ assay. CD30 fused to the Fc region of IgG1 (R&D Systems) was immobilized on a Protein A SPR chip, the surface was blocked with Fc, and WT and variant AC10 antibodies were flowed over the chip at a range of concentrations. The resulting sensorgrams are shown in Figure 15. Global Langmuir fits were carried out for the concentrations series using the BiaEvaluation curve fitting software, providing the on-rate constant (ka), off-rate constant (kd), and equilibrium binding constant (KD=kd/ka) for the curves. Table 3 provides the KDs and Fold KDs relative to WT for the SPR data. The excellent agreement between the rank ordering of the variants as determined by SPR and AlphaScreen™ assay support the accuracy of the data.

Table 3. CD30 Binding of AC10 Variants

AC10 Variant	SPR KD (nM)	SPR Fold KD	AlphaScreen IC50 (nM)	AlphaScreen Fold IC50
H2L1	9.49	0.36	55.1	0.06
H2L2	5.95	0.57	49.2	0.06
H1L2	7.55	0.45	45.3	0.07
H1L1	5.63	0.60	27.7	0.11
H2L3	6.75	0.50	27.2	0.12
H2L0	8.00	0.42	19.4	0.16
H1L3	5.09	0.67	17.4	0.18
H1L0	6.39	0.53	9.77	0.32
H0L2	3.48	0.97	7.81	0.41
H3L2	2.86	1.19	6.57	0.48
H3L0	3.08	1.10	6.18	0.51
H3L1	2.44	1.39	6.09	0.52
H0L1	3.29	1.03	5.19	0.61
H0L3	3.00	1.13	4.61	0.69
H0L0	3.39	1.00	3.18	1.00
H3L3	2.33	1.45	1.99	1.59

[180] In addition to assessing the antigen affinity and biophysical properties of the variants of the present invention, they may also be tested for effector functions in the context of a full length antibody. One advantage of generating multiple reduced immunogenicity variants of a parent immunoglobulin is that it enables a greater degree of sequence diversity to be sampled, diversity which may provide optimal properties. Some sequences may have more optimal properties than others, for example with regard to effector function. For example, as disclosed in USSN 60/614,944, and USSN 60/619,409, filed October 14, 2004, entitled "Immunoglobulin Variants Outside the Fc Region with Optimized Effector Function", the variable region of an antibody may impact effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP), and complement dependent cytotoxicity (CDC).

[181] In order to explore any differences in capacity to mediate effector function, the affinities of the AC10 variants for FcγRIIIa were measured using the AlphaScreen™ assay. The extracellular region of human V158 FcγRIIIa was obtained by PCR from a clone obtained from the Mammalian Gene Collection (MGC:22630), and the receptor was fused with glutathione S-Transferase (GST) to enable screening. Tagged FcγRIIIa was transfected in 293T cells, and media containing secreted FcγRIIIa were harvested and purified. The AlphaScreen™ assay was applied as a competition assay for screening AC10 variants for binding to FcγRIIIa. Biotinylated WT AC10 antibody was bound to streptavidin

donor beads (Perkin Elmer), and GST-fused human V158 FcγRIIIa was bound to anti-GST acceptor beads (Perkin Elmer). The binding data are shown in Figures 16a and 16b, and the resulting IC50's and Fold IC50's relative to WT are provided in Table 4. FcγRIIIa affinity of the AC10 variants was also measured using SPR. GST-fused human FcγRIIIa (V158 isoform) was immobilized on a chip, and WT and variant AC10 antibodies were flowed over the chip at a range of concentrations. Binding constants were obtained from fitting the data using standard curve-fitting methods. The equilibrium dissociation constants (KDs) obtained from the fits to these binding curves, and the calculated fold improvement or reduction relative to WT (Fold KD) are shown in Table 4.

Table 4. FcγRIIIa Binding of AC10 Variants

AC10 Variant	SPR KD (nM)	SPR Fold KD	AlphaScreen IC50 (nM)	AlphaScreen Fold IC50
H2L1	14.9	1.25	751	0.12
H2L2	4.01	4.64	146	0.60
H1L2	1.6.6	1.12	340	0.26
H1L1	11.2	1.66	221	0.39
H2L3	3.52	5.28	183	0.48
H2L0	12.9	1.44	175	0.50
H1L3	11.2	1.66	178	0.49
H1L0	22.0	0.85	71.6	1.22
H0L2	9.09	2.05	93.8	0.93
H3L2	3.57	5.21	88.7	0.98
H3L0	20.0	0.93	216	0.40
H3L1	17.4	1.07	209	0.42
H0L1	11.6	1.60	183	0.48
H0L3	12.7	1.46	146	0.60
H0L0	18.6	1.00	87.2	1.00
H3L3	6.13	3.03	83.5	1.04

[182] To assess the capacity of the AC10 variants to mediate effector function against CD30 expressing cells, the AC10 variants were tested in a cell-based ADCC assay. Human peripheral blood monocytes (PBMCs) were isolated from buffy-coat and used as effector cells, and CD30 positive L540 Hodgkin's lymphoma cells were used as target cells. L540 target cells were seeded at 20,000 per well in 96-well plates and treated with designated antibodies in triplicates starting at 1 μg/ml and in reduced concentrations in ½ log steps. PBMCs isolated using a Ficoll gradient and allotyped as FcγRIIIa 158 V/F were added at 25-fold excess of L540 cells and co-cultured for 4 hrs before processing for LDH activity using the Cytotoxicity Detection Kit (LDH, Roche Diagnostic Corporation, Indianapolis, IN) according to the manufacturer's instructions. The plates were read using a Wallac 1420

Victor^{2} ™. Figures 17a – 17c show the results. The graphs show that the antibodies differ not only in their EC50, reflecting their relative potency, but also in the maximal level of ADCC attainable by the antibodies at saturating concentrations, reflecting their relative efficacy. These two terms, potency and efficacy, are sometimes used loosely to refer to desired clinical properties. In the current experimental context, however, they are denoted as specific quantities, and therefore are here explicitly defined. By "potency" as used in the current experimental context is meant the EC50 of an EGFR targeting protein. By "efficacy" as used in the current experimental context is meant the maximal possible effector function of an antibody at saturating levels. Differences in capacity to mediate ADCC may be due to differences in antigen affinity, different capacities of the variant variable regions to effect FcγR binding, or both. Regardless, the contribution of an antibody variable region to FcγR binding and effector function may be an important parameter for selecting a clinical candidate. The choice of an antibody clinical candidate based in whole or in part on the impact on effector function of the variable region represents a novel dimension in antibody therapeutics.

[183] Based on the CD30 binding, FcγRIIIa binding, and ADCC results, the H3/L3 AC10 variant was chosen as a potential biotherapeutic candidate. Because this antibody is intended for clinical use as an anti-cancer therapeutic, it may be advantageous to optimize its effector function. As previously described, substitutions can be engineered in the constant region of an antibody to provide favorable clinical properties. In a most preferred embodiment, one or more amino acid modifications that provide optimized binding to FcγRs and/or enhanced effector function described in USSN 10/672,280, PCT US03/30249, and USSN 10/822,231, and USSN 60/627,774, filed 11/12/2004 and entitled "Optimized Fc Variants", are combined with the AC10 variants of the present invention. A number of optimized Fc variants obtained from these studies, including I332E, S239D, V264I/I332E, S239D/I332E, and S239D/A330L/I332E, were constructed in the H0/L0 and H3/L0 AC10 antibodies using quick change mutagenesis (Stratagene). Antibodies were expressed and purified as described above. Figures 18a and 18b show the results of the ADCC assay, carried out as described above, comparing WT (H0/L0) and H3/L3 AC10 in combination with the optimized Fc variants. Considerable enhancements in potency and efficacy are observed for the Fc variant antibodies as compared to H0/L0 and H3/L3 AC10.

[184] As described above, because variant sequences of the invention are preferably derived from a HSC-increasing procedure in which substitution of structurally important positions is disallowed (or discouraged), it is likely that additional optimization of HSC is possible if those positions are allowed to vary in a secondary analysis. It is noted that, due

to residue masking, mutations in the variants occur distal to the CDRs and VL/VH interface. This is in contrast to CDR grafted antibodies, which have mutations in the parent that are at or near these critical regions and thus have a significantly greater potential for perturbing antigen affinity. This is corroborated by the fact that CDR grafted antibodies typically require backmutations to the donor sequence to regain WT affinity for antigen. Such backmutations are usually made out of structural and immunogenic context with respect to host sequences, and cause dramatic reductions in the host string content of the final variant. In contrast, the variants presented herein are simultaneously optimized for host string and structural fitness within the same context, and no backmutations need be made. Nonetheless, one or more subsequent substitutions may be explored to increase antigen affinity or further improve HSC, for example by mutating residues that were masked in the calculations and/or residues in or close to the CDRs or VL/VH interface. Thus the H3/L3 variant can be thought of as a primary variant or template for further optimization, and variants of H3/L3 can be thought of as secondary variants. In contrast to backmutating as with CDR grafted antibodies, secondary substitutions in the variants of the present invention will comprise forward or neutral mutations with respect to the host germline, and thus are expected to only improve or unaffected HSC. An additional benefit of generating secondary variants is that, by exploring quality structural and string diversity, it is also possible that other properties can be optimized, for example affinity, activity, specificity, solubility, expression level, and effector function.

[185] String analysis was carried out on the H3/L3 sequence to design a set of secondary substitutions that have neutral, positive, or minimal impact on HSC, and/or that have significant potential for optimization of antigen affinity and/or effector function. Table 5 provides this set of 70 VL (Table 5a) and 64 VH (Table 5b) single mutations. The H3 column provides the WT H3 amino acid, and the Sub column provides the designed substitution. Positions are numbered according to the Kabat numbering format, with Kabat CDR positions bolded. The provided string impact, defined according to Equation 7, describes the difference in HSC between the primary variant sequence, here H3/L3, and the secondary variant sequence.

Table 5a. L3 AC10 Secondary Variants

Variant	Pos (Kabat)	L3	Sub	String Impact	Fold Prot A	Fold CD30	Fold FcγRIIIa
L3.1	1	D	A	0	0.89	1.30	0.96
L3.2	1	D	E	1	1.09	1.24	1.46

Variant	Pos (Kabat)	L3	Sub	String Impact	Fold Prot A	Fold CD30	Fold FcγRIIIa
L3.3	1	D	N	0	1.24	1.66	1.35
L3.4	1	D	S	0	0.97	1.04	1.18
L3.5	3	V	Q	0	1.13	1.32	1.32
L3.6	4	L	M	4	1.65	1.64	1.21
L3.7	**25**	A	S	6			
L3.8	**27a**	S	D	0	1.02	0.94	0.61
L3.9	**27b**	V	I	1	1.04	0.58	0.81
L3.10	**27c**	D	L	5			
L3.11	**27c**	D	S	8			
L3.12	**27c**	D	V	3	1.24	1.15	1.19
L3.13	**27d**	F	D	0	1.07	0.32	0.98
L3.14	**27d**	F	H	4	0.86	0.08	0.93
L3.15	**27d**	F	Y	5	1.04	0.63	1.19
L3.16	**28**	D	N	-1	1.10	1.61	1.18
L3.17	**30**	D	K	4	1.01	1.21	1.24
L3.18	**30**	D	N	6	1.09	1.45	0.94
L3.19	**30**	D	S	4	1.07	1.13	0.82
L3.20	**30**	D	Y	0	0.82	0.78	0.73
L3.21	**31**	S	D	0	1.01	0.81	0.95
L3.22	**31**	S	T	3	1.03	0.46	0.97
L3.23	**31**	S	N	-1	1.03	0.71	1.00
L3.24	**32**	Y	D	0	1.31	0.46	1.33
L3.25	**33**	M	L	8	1.38	1.36	1.37
L3.26	**34**	N	S	0			
L3.27	**34**	N	A	-1	1.39	0.38	1.36
L3.28	**34**	N	D	-6	1.19	0.41	1.76
L3.29	46	V	H	4	0.06	0.03	0.11
L3.30	46	V	L	9	0.86	0.39	0.75
L3.31	46	V	R	4			
L3.32	46	V	S	4	1.05	0.32	0.90
L3.33	**50**	A	D	6	0.98	0.26	0.66
L3.34	**50**	A	S	5	1.01	0.47	1.20
L3.35	**50**	A	W	2			
L3.36	**53**	N	S	8			
L3.37	**53**	N	T	5	0.99	1.26	1.01
L3.38	**54**	L	R	1	1.19	1.46	1.55
L3.39	**55**	E	A	2	1.01	0.85	1.32
L3.40	**55**	E	Q	6	0.99	0.87	1.07
L3.41	**56**	S	T	8	1.50	1.80	1.23
L3.42	58	I	V	4	1.44	1.55	0.95
L3.43	60	A	D	1	1.11	1.16	1.08
L3.44	60	A	S	2	0.82	1.08	0.85
L3.45	67	S	P	0			
L3.46	**89**	Q	H	1	1.37	0.08	1.64
L3.47	**91**	S	A	8			
L3.48	**91**	S	G	9	0.85	0.29	0.80
L3.49	**91**	S	H	2	1.20	0.01	1.32
L3.50	**91**	S	L	8	1.10	0.02	1.59

Variant	Pos (Kabat)	L3	Sub	String Impact	Fold Prot A	Fold CD30	Fold FcγRIIIa
L3.51	**91**	S	Y	8	1.00	0.02	1.50
L3.52	**92**	N	I	3			
L3.53	**92**	N	S	2	3.02	0.48	1.34
L3.54	**92**	N	Y	8	1.39	0.96	1.05
L3.55	**93**	E	K	8	0.62	0.27	0.49
L3.56	**93**	E	N	8	1.06	0.64	0.84
L3.57	**93**	E	Q	2			
L3.58	**93**	E	S	8	0.90	0.49	0.87
L3.59	**94**	D	A	3	1.16	0.09	1.14
L3.60	**94**	D	F	9	1.22	0.02	1.19
L3.61	**94**	D	H	8			
L3.62	**94**	D	L	3	0.87	0.46	0.79
L3.63	**94**	D	S	1	1.74	0.57	1.42
L3.64	**94**	D	T	7	1.24	0.14	1.16
L3.65	**96**	W	F		0.33	0.34	0.29
L3.66	**96**	W	I		0.75	0.00	0.57
L3.67	**96**	W	L				
L3.68	**96**	W	Y				
L3.69	100	G	P				
L3.70	100	G	Q				

Table 5b. H3 AC10 Secondary Variants

Variant	Position (Kabat)	H3	Sub	String Impact	Fold Prot A	Fold CD30
H3.1	1	Q	E	-1	0.83	1.00
H3.2	2	I	L	0	1.60	2.76
H3.3	2	I	M	2	0.88	0.68
H3.4	2	I	V	0	0.98	1.28
H3.5	9	P	A	2	0.95	1.29
H3.6	16	A	T	2	0.89	1.13
H3.7	24	A	V	2	1.54	4.45
H3.8	**31**	D	G	2	0.80	1.40
H3.9	**31**	D	S	2	0.82	1.65
H3.10	**33**	Y	D	2	0.68	0.07
H3.11	**33**	Y	G	3	0.96	0.73
H3.12	**33**	Y	W	0	0.84	0.00
H3.13	**34**	I	L	1	0.96	1.52
H3.14	**34**	I	M	8	1.05	1.62
H3.15	**35**	T	D	1	1.55	0.05
H3.16	**35**	T	G	2	1.03	0.15
H3.17	**35**	T	H	8	0.86	0.04
H3.18	**35**	T	N	4	1.07	0.13
H3.19	**35**	T	S	6	0.88	1.11
H3.20	44	G	A	0	1.20	2.04
H3.21	44	G	R	0	1.36	2.60
H3.22	**50**	W	I	6	1.25	0.01

Variant	Position (Kabat)	H3	Sub	String Impact	Fold Prot A	Fold CD30
H3.23	**50**	W	R	0	0.99	0.16
H3.24	**52**	Y	N	2	1.03	0.03
H3.25	**52**	Y	T	1	1.11	0.06
H3.26	**52**	Y	V	1	1.33	0.06
H3.27	**52a**	P	A	1	1.02	2.00
H3.28	**52a**	P	V	1	1.44	1.34
H3.29	**54**	S	D	1	1.45	1.81
H3.30	**54**	S	N	5	1.13	1.45
H3.31	**58**	K	G	4		
H3.32	**58**	K	I	2	1.22	1.09
H3.33	**58**	K	N	5	1.26	0.50
H3.34	**60**	N	A	7	0.87	1.30
H3.35	**60**	N	P	0		
H3.36	**60**	N	S	7	1.02	1.24
H3.37	**60**	N	T	0	1.12	1.01
H3.38	**60**	N	V	0	1.16	1.14
H3.39	**60**	N	D	0	1.09	1.00
H3.40	**61**	E	Q	7	1.51	1.83
H3.41	**64**	Q	T	0	0.98	1.38
H3.42	71	V	L	4	1.10	0.66
H3.43	71	V	M	9	1.17	0.88
H3.44	71	V	R	1	1.25	1.76
H3.45	87	T	M	-1	0.99	1.14
H3.46	89	V	M	-4	1.41	1.39
H3.47	91	F	H	2		
H3.48	91	F	Y	9	1.32	1.60
H3.49	93	A	T	0	1.47	0.38
H3.50	93	A	V	0	1.01	1.40
H3.51	94	N	A	9	1.51	0.08
H3.52	94	N	H	5	1.23	1.24
H3.53	94	N	K	9	1.67	0.02
H3.54	94	N	R	9	1.26	0.00
H3.55	94	N	T	9	1.24	0.91
H3.56	**99**	W	Y		1.26	0.07
H3.57	**101**	A	D		1.31	0.53
H3.58	**101**	A	Q		1.17	0.16
H3.59	**102**	Y	H		1.69	1.05
H3.60	**102**	Y	S		1.04	0.82
H3.61	**102**	Y	V		1.33	1.21
H3.62	**102**	Y	L		1.34	1.22
H3.63	**102**	Y	F		1.18	1.24
H3.64	105	Q	R		1.15	1.28

[186] The secondary H3/L3 variants were constructed using quick change mutagenesis, and the full length antibodies were expressed and purified as described above. H3 variants comprised H3 variant VH chains(H3.1- H3.64) in combination with L3 VL, and L3 variants comprised L3 variant VL chains (L3.1 – L3.70) in combination with H3 VH. The

AlphaScreen™ assay was used to measure binding of the H3/L3 secondary variants to CD30 and FcγRIIIa (as described earlier), as well as to protein A using biotinylated AC10 bound directly to protein A acceptor beads and streptavidin donor beads. Figure 19 provides AlphaScreen™ binding curves for binding of select AC10 variants to CD30. The Fold IC50's relative to WT H3/L3 for binding to CD30, FcγRIIIa, and protein A are provided in Table 5. A number of H3/L3 secondary variants provide comparable or improved binding to CD30 antigen relative to the H3/L3 parent, enabling the engineering of additional variants that comprise combinations of these substitutions, which may provide further enhancements in HSC and/or antigen affinity.

[187] Secondary substitutions that show favorable properties with respect to antigen affinity, effector function, stability, solubility, expression, and the like, may be combined in subsequent variants to generate a more optimized therapeutic candidate. Two new VL and three new VH variants were designed that comprise combinations of the described secondary substitutions, referred to as L3.71, L3.72, H3.68, H3.69, and H3.70. Figures 20 – 24 present the sequences, host string content, and mapped mutational differences on the modeled AC10 structure for each of these new AC10 VL and VH variants. Table 6 presents the number of mutations from the parent sequence, structural fitness scores, host string scores, and homology scores for these AC10 VL and VH variants.

Table 6. AC10 Variants

	L3.71	L3.72	H3.68	H3.69	H3.70
Mutations	15	15	23	27	30
Structural Consensus	0.56	0.55	0.46	0.46	0.45
Structural Precedence	0.54	0.52	0.55	0.57	0.56
Human String Content	0.88	0.87	0.80	0.83	0.84
Human String Similarity	0.52	0.45	0.39	0.47	0.47
Framework Region Homogeneity	0.47	0.51	0.33	0.40	0.42
N_9max	55	47	46	55	55
N_8max	19	24	26	26	34
N_7max	14	17	24	20	12
$N_{\leq 6}$max	19	19	23	18	18

	L3.71	L3.72	H3.68	H3.69	H3.70
Closest Germline	4-1	4-1	7-4-1	1-3	1-3
ID to Closest Germline	76 / 101 75%	76 / 101 75%	74 / 98 76%	77 / 98 79%	79 / 98 81%

[188] Because the provided AC10 variants antibodies are clinical candidates for anti-cancer therapeutics, it may be advantageous to optimize their effector function. As previously described, substitutions can be engineered in the constant region of an antibody to provide favorable clinical properties. Combinations of the variants of the present invention with Fc modifications that alter effector function are anticipated. In a most preferred embodiment, one or more amino acid modifications that provide optimized binding to FcγRs and/or enhanced effector function described in USSN 10/672,280, PCT US03/30249, and USSN 10/822,231, and USSN 60/627,774, filed 11/12/2004 and entitled "Optimized Fc Variants", are combined with the AC10 variants of the present invention. The optimal anti-CD30 clinical candidate may comprise amino acid modifications that reduce immunogenicity and enhance effector function relative to a parent anti-CD30 antibody. Figures 25a – 25c provide the light and heavy chain sequences of AC10 variants that comprise L3.71/H3.70 AC10 as described above, combined with a number of possible variant IgG1 constant regions, comprising one or more modifications at S239, V264, A330, and I332, that provide enhanced effector function.

[189] Although human IgG1 is the most commonly used constant region for therapeutic antibodies, other embodiments may utilize constant regions or variants thereof of other IgG immunoglobulin chains. Effector functions such as ADCC, ADCP, CDC, and serum half-life differ significantly between the different classes of antibodies, including for example human IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgD, IgE, IgG, and IgM (Michaelsen et al., 1992, Molecular Immunology, 29(3): 319-326). A number of studies have explored IgG1, IgG2, IgG3, and IgG4 variants in order to investigate the determinants of the effector function differences between them. See for example Canfield & Morrison, 1991, J. Exp. Med. 173: 1483-1491; Chappel et al., 1991, Proc. Natl. Acad. Sci. USA 88(20): 9036-9040; Chappel et al., 1993, Journal of Biological Chemistry 268:25124-25131; Tao et al., 1991, J . Exp. Med. 173: 1025-1028; Tao et al., 1993, J. Exp. Med. 178: 661-667; Redpath et al., 1998, Human Immunology, 59, 720-727. Using methods known in the art, it is possible to determine corresponding or equivalent residues in proteins that have significant sequence or structural homology with each other. By the same token, it is possible to use such methods to

engineer amino acid modifications in an antibody or Fc fusion that comprise constant regions from other immunoglobulin classes, for example as described in USSN 60/621,387 and 60/629,068, to provide optimal properties. As an example, the relatively poor effector function of IgG2 may be improved by replacing key FcγR binding residues with the corresponding amino acids in an IgG with better effector function, for example IgG1. For example, key residue differences between IgG2 and IgG1 with respect to FcγR binding may include P233, V234, A235, -236 (referring to a deletion in IgG2 relative to IgG1), and G327. Thus one or more amino acid modifications in the parent IgG2 wherein one or more of these residues is replaced with the corresponding IgG1 amino acids, P233E, V234L, A235L, -236G (referring to an insertion of a glycine at position 236), and G327A, may provide enhanced effector function. Furthermore, one or more additional amino acid modifications, for example the S239D, V264I, A330L, I332E, or combinations thereof as described above, may provide enhanced FcγR binding and effector function relative to the parent IgG2. Figures 25a, 25d, and 25e illustrate this embodiment, providing the light and heavy chain sequences of AC10 variants that comprise L3.71/H3.70 AC10 combined with a number of possible variant IgG2 constant regions.

[190] The Fc modifications defined in Figure 25 that provide enhanced effector function are not meant to constrain the invention to only these modifications for effector function optimization. For example, as described in US 6,737,056, PCT US2004/000643, USSN 10/370,749, and PCT/US2004/005112, the substitutions S298A, S298D, K326E, K326D, E333A, K334A, and P396L provide optimized FcγR binding and/or enhanced ADCC. Furthermore, as disclosed in Idusogie et al., 2001, J. Immunology 166:2571-2572, substitutions K326W, K326Y, and E333S provide enhanced binding to the complement protein C1q and enhanced CDC. As described in Hinton et al., 2004, J. Biol. Chem. 279(8): 6213-6216, substitutions T250Q, T250E, M428L, and M428F provide enhanced binding to FcRn and improved pharmacokinetics. Modifications need not be restricted to the Fc region. It is also possible that the mutational differences in the Fab and hinge regions may provide optimized FcγR and/or C1q binding and/or effector function. For example, as disclosed in USSN 60/614,944, and USSN 60/619,409, filed October 14, 2004, entitled "Immunoglobulin Variants Outside the Fc Region with Optimized Effector Function", the Fab and hinge regions of an antibody may impact effector functions such as antibody dependent cell-mediated cytotoxicity (ADCC), antibody dependent cell-mediated phagocytosis (ADCP), and complement dependent cytotoxicity (CDC). Thus immunoglobulin variants comprising substitutions in the Fc, Fab, and/or hinge regions are contemplated. For example, the antibodies may be combined with one or more substitutions in the VL, CL, VH, CH1, and/or

hinge regions. Furthermore, further modifications may be made in non IgG1 immunoglobulins to corresponding amino acids in other immunoglobulin classes to provide more optimal properties, as described in USSN 60/621,387, filed 10/21/2004, entitled "IgG Immunoglobulin Variants with Optimized Effector Function". For example, in one embodiment, an IgG2 antibody, similar to the antibody presented in Figure 25, may comprise one or more modifications to corresponding amino acids in IgG1 or IgG3 CH1, hinge, CH2, and/or CH3. In another embodiment, an IgG2 antibody, similar to the antibody presented in Figure 25, may comprise all of the IgG1 CH1 and hinge substitutions,. i.e. said IgG2 variant comprises the entire CH1 domain and hinge of IgG1.

Example 2. Immunogenicity Reduction of C225

[191] To illustrate further application of the method described in the present invention, and to validate its broad applicability to immunogenicity reduction of proteins, a second xenogeneic antibody example is provided using the variable region of C225 as the parent sequence (cetuximab, Erbitux®, Imclone) (US 4,943,533; PCT WO 96/40210). C225 is a murine anti-EGFR antibody, a chimeric version of which is currently approved for the treatment of cancer. A structural model of the murine C225 variable region was constructed using standard antibody modeling methods known in the art. Figures 26 and 27 show the sequences, host string content, and structures of the C225 VL and VH domains. A CDR graft of this antibody was constructed by placing the C225 CDRs into the context of the frameworks of the most homologous human germlines, determined to be vlk_6D-21 for VL and vh_4-30-4 for VH using the sequence alignment program BLAST. The sequences and string content of these CDR grafts are shown in Figures 28 and 29, along with structures of modeled C225 highlighting the mutational differences between the CDR grafted C225 variable chains and WT.

[192] Variants with reduced immunogenicity were generated by applying a string optimization algorithm on the WT C225 VL and VH sequences, similar to as described above for AC10 except that single instead of multiple amino acid substitutions were sampled. HSC of each sequence was optimized using a window size w=9, and the same set of CDR and VL/VH interface proximal residues were masked. The calculation was run for C225 VL and VH in 100 separate iterations, generating a set of diverse C225 variants with more host string content than WT. Figure 30 shows the nonredundant set of output sequences from these calculations for the C225 VL and VH regions, referred to as C225 VL HSC Calculation 1 and C225 VH HSC Calculation 1, respectively. In addition to the HSC score, the structural consensus and structural precedence of each sequence was evaluated (USSN 60/528,229,

filed December 8, 2003, entitled Protein Engineering with Analogous Contact Environments) in order to evaluate its structural integrity.

[193] A second set of similar calculations were run on the C225 VL and VH sequences, except that the algorithm was allowed to sample multiple amino acid substitutions, rather than only single substitutions, in order to optimize HSC. Figure 31 shows the nonredundant set of output sequences from these calculations for the C225 VL and VH regions, referred to as C225 VL HSC Calculation 2 and C225 VH HSC Calculation 2, respectively. Here, two measure of structural fitness, referred to as "Structural Consensus" and "Structural Precedence" (USSN 60/528,229 and USSN 60/602,566), are used to evaluate the structural and functional integrity of the sequences, in addition to HSC score. The output sequences were clustered based on their mutational distance from the other sequences in the set, and these clusters are delineated by the horizontal black lines in the Figure.

[194] The calculations described above and presented in Figures 30 and 31 were used to generate a set of C225 VL and VH variants. In some cases, further substitutions were made to output sequences, using string and structural scores, as well as visual inspection of the modeled C225 structure, to evaluate fitness. Figures 32 - 40 present the sequences, structural scores, string scores, and mapped mutational differences on the modeled C225 structure for each of the C225 VL and VH variants. Iteration 21 from C225 VL HSC calculation 1 served as the precursor for L2 C225 VL, iteration 17 from C225 VL HSC calculation 2 served as the precursor for L3 C225 VL, and iteration 38 from C225 VL HSC calculation 2 served as the precursor for L4 C225 VL. Iteration 23 from C225 VH HSC calculation 1 served as the precursor for H3, H4, and H5 C225 VH, iteration 5 from C225 VH HSC calculation 2 served as the precursor for H6 C225 VH, iteration 41 from C225 VH HSC calculation 2 served as the precursor for H7 C225 VH, and iteration 44 from C225 VH HSC calculation 2 served as the precursor for H8 C225 VH.

[195] Tables 7 and 8 present the mutational, structural fitness, and host string content scores for the C225 VL and VH variants as compared to the WT and CDR grafted C225 sequences. In addition, the maximum identity match to the germline for each string in the sequences was also determined, referred to as $N_{ID}max$. This represents the total number of strings in each sequence whose maximum identity to the corresponding strings in the human germline is the indicated value. For w = 9, Tables 7 and 8 list N_9max, N_8max, N_7max, and $N_{\geq 6}max$ for each sequence. Also provided is the framework region homogeneity. In addition to the aforementioned structural and host string analysis, each sequence was analyzed for its global homology to the human germline; tables 7 and 8 present the most homologous

human germline sequence for each sequence (Closest Germline) and corresponding identity to that germline (ID to Closest Germline), determined using the sequence alignment program BLAST.

Table 7. C225 VL Variants

	WT	CDR Graft	L2	L3	L4
Mutations		25	17	21	18
Structural Consensus	0.49	0.52	0.56	0.58	0.54
Structural Precedence	0.53	0.56	0.57	0.59	0.57
Human String Content	0.79	0.94	0.91	0.92	0.91
Human String Similarity	0.15	0.65	0.51	0.58	0.57
Framework Region Homogeneity		0.97	0.52	0.50	0.78
N_9max	13	69	52	60	58
N_8max	27	15	28	24	23
N_7max	37	22	20	16	19
$N_{\leq 6}$max	30	1	7	7	7
Closest Germline	6D-21	6D-21	3-11	1D-13	6D-21
ID to Closest Germline	63 / 95 66%	87 / 95 91%	72 / 95 75%	73 / 94 77%	79 / 95 83%

Table 8. C225 VH Variants

	WT	CDR Graft	H3	H4	H5	H6	H7	H8
Mutations		33	18	21	15	21	22	28
Structural Consensus	0.44	0.48	0.51	0.46	0.49	0.52	0.49	0.53
Structural Precedence	0.55	0.54	0.55	0.54	0.51	0.55	0.58	0.55
Human String Content	0.67	0.84	0.79	0.81	0.77	0.79	0.79	0.79
Human String Similarity	0.04	0.56	0.36	0.41	0.33	0.36	0.35	0.33
Framework Region Homogeneity		0.97	0.45	0.52	0.50	0.50	0.76	0.77
N_9max	3	66	42	48	38	42	41	39
N_8max	23	17	25	24	17	24	21	27
N_7max	32	10	19	16	26	23	25	23

	WT	CDR Graft	H3	H4	H5	H6	H7	H8
$N_{\leq}$max	61	26	33	31	38	30	32	30
Closest Germline	4-30-4	4-30-4	4-30-4	2-26	4-30-4	3-33	4-30-4	3-33
ID to Closest Germline	56 / 99 56%	88 / 99 88%	67 / 99 66%	74 / 99 74%	64 / 99 64%	69 / 99 70%	67 / 99 67%	80 / 99 81%

[196] Again, whereas the CDR grafted C225 antibodies are most homologous to a single human germline sequence, the C225 variants of the present invention are homologous to different human germline sequences in different regions of the sequence. Whereas CDR grafted C225 VH is most homologous to human germline subfamily 4 across its entire sequence, H4 C225 VH is most homologous to subfamily 4 in FR1, subfamily 3 in FR2, and subfamily 2 in FR3. Additionally, whereas the CDR grafted antibodies are most homologous to a single germline sequence that is also the most homologous sequence to the parent sequence, the present invention presents a set of antibodies for a given antibody that are most homologous to different human germline sequences, which need not be the most homologous germline sequence to WT. For example, Table 7 shows that CDR grafted C225 VL is most homologous to vlk_6D-21, which is also the most homologous human germline to WT C225. However L2, L3, and L4 are most homologous to three different human germlines - vlk_3-11, vlk_1D-13, and vlk_6D-21 respectively. Thus the variants of the present invention explore a substantially greater amount of diversity than CDR grafted antibodies.

[197] The genes for the C225 variable regions were constructed as described above, and subcloned into a modified pASK84 vector (Skerra, 1994, Gene 141: 79-84) comprising mouse constant regions for expression as Fabs. Select C225 variants were experimentally tested for their capacity to bind EGFR antigen. L2/H3 and L2/H4 C225 Fabs were expressed from the pASK84 vector in *E. Coli* with a His-tag, and purified using Nickel-affinity chromatography. Antigen affinity of the C225 variants was tested using SPR similar to as described above. EGFR extracellular domain (purchased commercially from R&D Systems) was covalently coupled to the dextrane matrix of a CM5 chip using NHS-linkage chemistry. C225 Fabs were reacted with the EGFR sensor chip surface at varying concentrations. Global Langmuir fits were been carried out for the concentrations series using the BiaEvaluation curve fitting software. The on-rate constant (ka), off-rate constant (kd), equilibrium binding constant (KD=kd/ka), and predicted saturation binding signal (Rmax) derived from these fits are presented in Table 9, along with the Chi2 which quantifies the

average deviation of the fit curve from the actual data curve. The data indicate that both the L2/H3 and L2/H4 C225 variants bind EGFR antigen.

Table 9. SPR data on C225 Variants

C225	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi2
L2/H3	2.79×10^{4}	5.35×10^{-3}	1.92×10^{-7}	174	8.83
L2/H4	1.79×10^{4}	4.73×10^{-3}	2.64×10^{-7}	153	2.69

[198] In order to investigate the anti-EGFR variants in the context of a full length antibody, the C225 WT (L0 ad H0) and variant (L2, L3, L4, H3, H4, H5, H6, H7, and H8) regions were subcloned into the mammalian expression vector pcDNA3.1Zeo (Invitrogen) as described above. All combinations of the light and heavy chain plasmids were co-transfected into 293T cells, and antibodies were expressed, harvested, and purified as described above. Binding of the C225 WT (L0/H0) and variant (L0/H3, L0/H4, L0/H5, L0/H6, L0/H7, L0/H8, L2/H3, L2/H4, L2/H5, L2/H6, L2/H7, L2/H8, L3/H3, L3/H4, L3/H5, L3/H6, L3/H7, L3/H8, L4/H3, L4/H4, L4/H5, L4/H6, L4/H7, and L4/H8) antibodies was determined using SPR similar to as described above. Full length antibodies were flowed over the EGFR sensor chip described above. Figure 41 shows the SPR sensorgrams obtained from the experiments. The curves consist of a association phase and dissociation phase, the separation being marked by a little spike on each curve. As a very rough approximation the signal level reached near the end of the association phase can be used as an indicator for relative binding. For all the curves this signal level is within 25% of the average level indicating that none of the antibody variants have significantly lost their ability to bind to EGFR.

[199] To assess the capacity of the anti-EGFR antibodies to mediate effector function against EGFR expressing cells, the C225 variants were tested in a cell-based ADCC assay. Human peripheral blood monocytes (PBMCs) were used as effector cells, A431 epidermoid carcinoma cells were used as target cells, and lysis was monitored by measuring LDH activity using the Cytotoxicity Detection Kit as described above. Figure 42 shows the dose dependence of ADCC at various antibody concentrations for WT and variant C225 antibodies. The results show that a number of the C225 variants have comparable or better ADCC than WT C225 with respect to potency and efficacy. These data may be weighed together with the antigen affinity data and other data to choose the optimal anti-EGFR clinical candidate. As exemplified above with AC10 variants, combinations of the C225 variants of the present invention with amino acid modifications that alter effector function are

contemplated.

Example 3. Immunogenicity Reduction of ICR62

[200] To further illustrate application of the method described in the present invention, and to validate its broad applicability to immunogenicity reduction of proteins, an example is provided using as the parent sequence the anti-EGFR antibody ICR62 (Institute of Cancer Research) (PCT WO 95/20045; Modjtahedi et al., 1993, *J. Cell Biophys.* 1993, 22(1-3):129-46; Modjtahedi et al., 1993, *Br J Cancer.* 1993, 67(2):247-53; Modjtahedi et al, 1996, *Br J Cancer*, 73(2):228-35; Modjtahedi et al, 2003, *Int J Cancer,* 105(2):273-80). A structural model of the rat ICR62 variable region was constructed using standard antibody modeling methods known in the art. Figures 43 and 44 show the sequences, host string content, and structures of the ICR62 VL and VH domains. A CDR graft of this antibody was constructed by placing the ICR62 CDRs into the context of the frameworks of the most homologous human germlines, determined to be vlk_1-17 for VL and vh_1-f for VH using the sequence alignment program BLAST. The sequences and string content of these CDR grafts are shown in Figures 45 and 46, along with structures of modeled ICR62 highlighting the mutational differences between the CDR grafted ICR62 variable chains and WT.

[201] Variants with reduced immunogenicity were generated by applying a string optimization algorithm on the WT ICR62 VL and VH sequences, similar to as described above for AC10 except that single instead of multiple amino acid substitutions were sampled. HSC of each sequence was optimized using a window size w=9, and the same set of CDR and VL/VH interface proximal residues were masked. The calculation was run for ICR62 VL and VH in 100 separate interactions, generating a set of diverse ICR62 variants with more host string content than WT. Figure 47 shows the nonredundant set of output sequences from these calculations for the ICR62 VL and VH regions, referred to as ICR62 VL HSC Calculation 1 and ICR62 VH HSC Calculation 1 respectively. In addition to the HSC score, the structural consensus and structural precedence of each sequence was evaluated (USSN 60/528,229, filed December 8, 2003, entitled Protein Engineering with Analogous Contact Environments) in order to evaluate its structural integrity.

[202] The calculations described above and presented in Figure 47 were used to generate a set of ICR62 VL and VH variants. In some cases, further substitutions were made to output sequences, using HSC and Structural Precedence scores, as well as visual inspection of the modeled ICR62 structure, to evaluate fitness. Figures 48 – 50 present the sequences, host string content, and mapped mutational differences on the modeled ICR62 structure for each of the ICR62 VL and VH variants. Iteration 20 from ICR62 VL HSC

calculation 1 served as the precursor for L2 ICR62 VL. Iteration 1 from ICR62 VH HSC calculation 1 served as the precursor for H9, and iteration 5 from ICR62 VH HSC calculation 2 served as the precursor for H10 ICR62 VH.

[203] Tables 10 and 11 present the mutational, structural fitness, and host string content scores for the ICR62 VL and VH variants as compared to the WT and CDR grafted ICR62 sequences. In addition, the maximum identity match to the germline for each string in the sequences, referred to as N_{ID}max, is also provided, as well as the framework region homogeneity. In addition to the aforementioned structural and host string analysis, each sequence was analyzed for its global homology to the host germline; tables 10 and 11 present the most homologous host germline sequence for each sequence (Closest Germline) and corresponding identity to that germline (ID to Closest Germline), determined using the sequence alignment program BLAST.

Table 10. ICR62 VL Variants

	WT	CDR Graft	L2
Mutations	0	11	6
Structural Consensus	0.56	0.60	0.61
Structural Precedence	0.52	0.58	0.57
Human String Content	0.86	0.91	0.90
Human String Similarity	0.38	0.58	0.56
Framework Region Homogeneity	0.62	0.97	0.64
N_9max	37	59	56
N_8max	26	21	19
N_7max	31	18	22
$N_{\leq 6}$max	13	9	10
Closest Germline	1-17	1-17	1-17
ID to Closest Germline	76 / 95 80%	86 / 95 90%	81 / 95 85%

Table 11. ICR62 VH Variants

	WT	CDR Graft	H9	H10
Mutations	0	34	20	21

	WT	CDR Graft	H9	H10
Structural Consensus	0.43	0.44	0.46	0.45
Structural Precedence	0.42	0.52	0.47	0.49
Human String Content	0.64	0.85	0.79	0.79
Human String Similarity	0.01	0.54	0.28	0.33
Framework Region Homogeneity		1.00	0.64	0.85
N_9max	1	64	33	39
N_8max	16	24	33	30
N_7max	35	14	28	25
$N_{\leq 6}$max	67	17	25	25
Closest Germline	1-f	1-f	1-f	1-f
ID to Closest Germline	60 / 98 61%	92 / 98 93%	72 / 98 73%	77 / 98 79%

[204] Again, as observed from the significant differences in FRH and closest germlines, the ICR62 variants are homologous to different host germline sequences in different regions of the sequence. The genes for the ICR62 WT and L2/H9 variable regions were constructed as described above, and subcloned into a modified pASK84 vector (Skerra, 1994, Gene 141: 79-84). The ICR62 Fabs experimentally tested for their capacity to bind EGFR antigen. WT and L2/H9 ICR62 Fabs were expressed from the pASK84 vector in *E. Coli* with a His-tag, and purified using Nickel-affinity chromatography. Antigen affinity of the ICR62 antibodies was tested using SPR similar to as described above, with EGFR covalently coupled to the CM5 chip reacted with ICR62 antibodies at varying concentrations. The fits to the data, as described above, are provided in Table 12. fits were been carried out for the concentrations series using the BiaEvaluation curve fitting software. As can be seen, L2/H9 ICR62 binds with comparable affinity as WT to the EGFR antigen.

Table 12. SPR data on ICR62 Variants

ICR62	ka (1/Ms)	kd (1/s)	KD (M)	Rmax (RU)	Chi2
WT	9.86×10^4	2.53×10^{-5}	2.57×10^{-10}	402	1.86
L2/H9	2.35×10^5	1.06×10^{-4}	4.50×10^{-10}	508	4.91

Example 4. String diversity exploration of immunoglobulins

[205] The generation of mutational diversity based on HSC is much broader than the primary variant – secondary variant strategy described above for H3/L3 AC10. Indeed substitutions can be designed for any parent protein wherein the substitutions result in positive or neutral impact on the host string content of the parent sequence. Again, the advantage of such a strategy is that it generates a diverse set of minimally immunogenic variants that have the potential for optimized properties, including but not limited to antigen affinity, activity, specificity, solubility, expression level, and effector function. Such a set of variants may be designed, for example, to explore diversity for other parent immunoglobulins, including but not limited to nonhuman antibodies, humanized or otherwise engineered antibodies (Clark, 2000, *Immunol Today* 21:397-402), (Tsurushita & Vasquez, 2004, Humanization of Monoclonal Antibodies, Molecular Biology of B Cells, 533-545, Elsevier Science (USA)), and "fully human" antibodies, obtained for example using transgenic mice (Bruggemann *et al.*, 1997, *Curr Opin Biotechnol* 8:455-458) or human antibody libraries coupled with selection methods (Griffiths *et al.*, 1998, *Curr Opin Biotechnol* 9:102-108).

Example 5. Unique properties of variant proteins generated by the methods of the present invention

[206] The methods described in the present invention generate variant proteins that possess a number of unique properties relative to variant proteins generated by other methods that attempt to achieve the same or similar goal. Figures 51 and 52 provide the host string content (HSC, Equation 3), exact string content (ESC, Equation 3a), and framework region homogeneity (FRH, Equation 10) of the AC10, C225, and ICR62 VH (Figure 51) and VL (Figure 52) variants of the present invention, compared with a number of antibody variable regions "humanized" by methods in the prior art. If a variant sequence's exact string content is derived solely from a single germline sequence, the FRH would be close to 1.0. Alternatively, as is the case with many of the variant sequences created by the present invention, FRH values can be significantly less than 1, with values ranging from 0.4 to 1.0, indicating, as expected, that sequences with high exact string content can be discovered with contributions from multiple germline subfamilies and sequences. At the same time, the variant sequences engineered using the present invention have high host string content, and thus are predicted to have low potential for immunogenicity in humans. For example, as shown in Figure 51 variant VH sequences generated using the present invention have HSC values generally higher than 75%, and many of them have FRH values

lower than 0.6, indicating their HSC is derived from multiple germline frameworks. As shown in Figure 52, similar trends apply for variant VL sequences generated using the present invention

[207] Whereas particular embodiments of the invention have been described above for purposes of illustration, it will be appreciated by those skilled in the art that numerous variations of the details may be made without departing from the invention as described in the appended claims. All references are herein expressly incorporated by reference.

CLAIMS

We claim:

1. A method of generating a variant protein for a host as compared to a parent protein, comprising:

A. comparing said parent protein sequence with two or more natural protein sequences from said host species;

B. analyzing one or more amino acid strings of said parent protein sequence with a corresponding amino acid string of each natural protein sequence; and,

C. substituting one or more amino acids of said parent protein sequence with a corresponding amino acid string of a natural protein sequence on an amino acid string by amino acid string basis.

D. wherein said variant protein has increased host string content as compared to said parent protein, and,

E. wherein said substituted amino acids include a first substitution in a first string from a first natural protein, and a second substitution in a second string from a second natural protein.

2. The method according to claim 1, wherein said substituted amino acid is a consensus of two or more natural proteins.

3. The method according to claim 1, wherein said host species is human.

4. The method according to claim 1, wherein said parent protein family is murine.

5. The method according claim 1, wherein said natural protein sequences are germline sequences.

6. The method according to claim 1, wherein one of the substitutions is not the corresponding residue from the most homologous natural sequence.

7. The method according to claim 5, wherein one of the substitutions is not the corresponding residue from a consensus of homologous natural sequences.

8. The method according to claim 1, wherein one of the substitutions is made at a position that is not surface exposed.

9. The method according to claim 1, wherein said variant protein is an antibody.

10. The method according to claim 9, wherein one of the substitutions is not in a CDR.

11. The method according to claim 9, wherein one of the substitutions is made at a position that is part of the VH/VL interface.

12. The method according to claim 9, wherein said variant antibody comprises an affinity for an antigen no lower than about two-fold less than said parent protein.

13. The method according to claim 9, wherein said variant antibody comprises an affinity for an antigen of about two-fold more than said parent protein.

14. The method according to claim 9, wherein said variant protein comprises a framework region identity as compared to a natural protein sequence of less than about 85%.

15. The method according to claim 9, wherein said variant protein comprises a framework region homogeneity of less than about 0.6.

16. The method according to claim 9, wherein said variant antibody comprises no substitutions within about 5 Angstroms of a CDR residue.

17. The method according to claim 9, wherein said antibody has modulated effector function as compared to said parent antibody.

18. The method according to claim 17, wherein said effector function is ADCC.

19. The method according to claim 1, wherein more than one variant protein is generated from said method to form a variant protein set.

20. The method according to claim 19, wherein said variant protein set comprises at least two variant proteins that differ by more than about 5 amino acids.

21. The method according to claim 19, further synthesizing at least one variant protein.

22. The method according to claim 1, wherein said analyzing one or more amino acid strings is not applied to a CDR region.

23. The method according to claim 1, wherein said variant protein has at least one improved property is selected from a group consisting of solubility, stability, expression, affinity, immunogenicity, activity and functionality.

24. The method according to claim 1, wherein said analyzing one or more amino acid strings includes theoretical binding to MHC agretopes.

25. The method according to claim 24, wherein said string is nine amino acids in length.

26. A computer readable memory to direct a computer to function in a specified manner comprising:

A. a comparison module to compare a parent protein sequence and two or more host natural protein sequences;

B. an analyzing module to provide a comparison between a parent protein sequence and one or more natural protein sequences;

C. a substitution module to replace one or more amino acids said parent protein sequence with a corresponding amino acid string of a natural protein sequence on an amino acid by amino acid basis,

D. wherein said variant protein has increased host string content as compared to said parent protein.

27. A method of generating a variant protein for a host as compared to a parent protein, comprising:

A. comparing said parent protein sequence with two or more natural protein sequences;

B. substituting amino acids in said parent protein sequence with amino acids in one or more natural protein sequences, wherein a substitution increases the sequence identity of said variant protein to a natural protein sequence as compared to said parent protein within a string,

C. wherein said variant protein has increased host string content as compared to said parent protein.

28. A method of generating a variant protein for a host as compared to a parent protein, comprising:

A. comparing said parent protein sequence with two or more natural protein sequences from said host species;

B. analyzing one or more amino acid strings of said parent protein sequence with a corresponding amino acid string of each natural protein sequence; and,

C. substituting one or more amino acids of said parent protein sequence with a corresponding amino acid string of a natural protein sequence on an amino acid string by amino acid string basis,

D. wherein said variant protein has increased host string content.

29. The method according to claim 28, wherein said substituted amino acid is a consensus of two or more natural proteins.

30. The method according to claim 28, wherein said host species is human.

31. The method according to claim 28, wherein said parent protein is murine.

32. The method according to claim 28, wherein said natural protein sequences are germline sequences.

33. The method according to claim 28, wherein one of the substitutions is not the corresponding residue from the most homologous natural sequence.

34. The method according to claim 33, wherein one of the substitutions is not the corresponding residue from a consensus of homologous natural sequences.

35. The method according to claim 28, wherein one of the substitutions is made at a position that is not surface exposed.

36. The method according to claim 28, wherein said variant protein is an antibody.

37. The method according to claim 36, wherein one of the substitutions is not in a CDR.

38. The method according to claim 36, wherein one of the substitutions is made at a position that is part of the VH/VL interface.

39. The method according to claim 36, wherein said variant antibody comprises an affinity for an antigen no lower than about two-fold less than said parent protein.

40. The method according to claim 36, wherein said variant antibody comprises an affinity for an antigen of about two-fold more than said parent protein.

41. The method according to claim 36, wherein said variant protein comprises a framework region identity as compared to a natural protein sequence of less than about 85%.

42. The method according to claim 36, wherein said variant protein comprises a framework region homogeneity of less than about 0.6.

43. The method according to claim 36, wherein said variant antibody comprises less than about 28 amino acid substitutions, as compared to a parent protein.

44. The method according to claim 36, wherein said variant antibody comprises no substitutions within about 5 Angstroms of a CDR residue.

45. The method according to claim 36, wherein said antibody has modulated effector function as compared to said parent antibody.

46. The method according to claim 45, wherein said effector function is ADCC.

47. The method according to claim 28, wherein more than one variant protein is generated from said method to form a variant protein set.

48. The method according to claim 47, wherein said variant protein set comprises at least two variant proteins that differ by more than about 5 amino acids.

49. The method according to claim 47, further synthesizing at least one variant protein.

50. The method according to claim 28, wherein said strings are not applied to a CDR region.

51. The method according to claim 28, wherein said variant protein has at least one improved property is selected from a group consisting of solubility, stability, expression, affinity, immunogenicity, activity and functionality.

52. The method according to claim 28, wherein said analyzing one or more amino acid strings includes theoretical binding to MHC agretopes.

53. The method according to claim 52, wherein said string is nine amino acids in length.

Figure 1a. Human Germline VH Sequences

Chain	FR1	CDR1	FR2	CDR2	FR3	CDR3
	1 2	3	4 5	6	7 8 9	10
Kabat	1234567890123456789012345678901234 5ab	6789012345678901 2abc	3456789012345678901234567890 12abc	3456789012345	67890abcdefghijk12	
VH_1-2	QVQLVQSGAEVKKPGASVKVSCKASGYTFT GYYMH	WVRQAPGQGLEWMGRINP	NSGGTNYAQKFQG	RVTSTRDTSISTAYMELSRLRSDDTVVYYCAR		
VH_1-3	QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYAMH	WVRQAPGQRLEWMGWINA	GNGNTKYSQKFQG	RVTITRDTSASTAYMELSSLRSEDTAVYYCAR		
VH_1-8	QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYDIN	WVRQATGQGLEWMGWMNP	NSGNTGYAQKFQG	RVTMTRNTSISTAYMELSSLRSEDTAVYYCAR		
VH_1-18	QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYGIS	WVRQAPGQGLEWMGWISA	YNGNTNYAQKLQG	RVTMTTDTSTSTAYMELRSLRSDDTAVYYCAR		
VH_1-24	QVQLVQSGAEVKKPGASVKVSCKVSGYTLT ELSMH	WVRQAPGKGLEWMGGFDP	EDGETIYAQKFQG	RVTMTEDTSTDTAYMELSSLRSEDTAVYYCAT		
VH_1-45	QMQLVQSGAEVKKTGSSVKVSCKASGYTFT YRYLH	WVRQAPGQALEWMGWITP	FNGNTNYAQKFQD	RVTITRDRSMSTAYMELSSLRSEDTAMYYCAR		
VH_1-46	QVQLVQSGAEVKKPGASVKVSCKASGYTFT SYYMH	WVRQAPGQGLEWMGIINP	SGGSTSYAQKFQG	RVTMTRDTSTSTVYMELSSLRSEDTAVYYCAR		
VH_1-58	QMQLVQSGPEVKKPGTSVKVSCKASGFTFT SSAVQ	WVRQARGQRLEWIGWIVV	GSGNTNYAQKFQE	RVTITRDMSTSTAYMELSSLRSEDTAVYYCAA		
VH_1-69	QVQLVQSGAEVKKPGSSVKVSCKASGGTFS SYAIS	WVRQAPGQGLEWMGGIIP	IFGTANYAQKFQG	RVTITADESTSTAYMELSSLRSEDTAVYYCAR		
VH_1-f	EVQLVQSGAEVKKPGATVKISCKVSGYTFT DYYMH	WVQQAPGKGLEWMGLVDP	EDGETIYAEKFQG	RVTITADTSTDTAYMELSSLRSEDTAVYYCAT		
VH_2-5	QITLKESGPTLVKPTQTLTLTCTFSGFSLS TSGVGVG	WIRQPPGKALEWLALIY	WNDDKRYSPSLKS	RLTITKDTSKNQVVLTMTNMDPVDTATYYCAHR		
VH_2-26	QVTLKESGPVLVKPTETLTLTCTVSGFSLS NARMGVS	WIRQPPGKALEWLAHIF	SNDEKSYSTSLKS	RLTISKDTSKSQVVLTMTNMDPVDTATYYCARI		
VH_2-70	QVTLRESGPALVKPTQTLTLTCTFSGFSLS TSGMCVS	WIRQPPGKALEWLALID	WDDDKYYSTSLKT	RLTISKDTSKNQVVLTMTNMDPVDTATYYCARI		
VH_3-7	EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYWMS	WVRQAPGKGLEWVANIKQ	DGSEKYYVDSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
VH_3-9	EVQLVESGGGLVQPGRSLRLSCAASGFTFD DYAMH	WVRQAPGKGLEWVSGISW	NSGSIGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYYCAKD		
VH_3-11	QVQLVESGGGLVKPGGSLRLSCAASGFTFS DYYMS	WIRQAPGKGLEWVSYISS	SGSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
VH_3-13	EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYDMH	WVRQATGKGLEWVSAIGT	AGDT YYPGSVKG	RFTISRENAKNSLYLQMNSLRAGDTAVYYCAR		
VH_3-15	EVQLVESGGGLVKPGGSLRLSCAASGFTFS NAWMS	WVRQAPGKGLEWVGRIKSKTDGGTTDYAAPVKG	RFTISRDDSKNTLYLQMNSLKTEDTAVYYCTT			
VH_3-16	EVQLVESGGGLVQPGGSLRLSCAASGFTFS NSDMN	WARKAPGKGLEWVSGVSW	NGSRTHYVDSVKR	RFIISRDNSRNSLYLQKNRRRAEDMAVYYCVR		
VH_3-19	TVQLVESGGGLVEPGGSLRLSCAASGFTFS NSDMN	WVRQAPGKGLEWVSGVSW	NGSRTHYADSVKG	RFIISRDNSRNFLYQQMNSLRPEDMAVYYCVR		
VH_3-20	EVQLVESGGGVVRPGGSLRLSCAASGFTFD DYGMS	WVRQAPGKGLEWVSGINW	NGGSTGYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTALYHCAR		
VH_3-21	EVQLVESGGGLVKPGGSLRLSCAASGFTFS SYSMN	WVRQAPGKGLEWVSSISS	SSSYIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
VH_3-23	EVQLLESGGGLVQPGGSLRLSCAASGFTFS SYAMS	WVRQAPGKGLEWVSAISG	SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAK		
VH_3-30	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYAMH	WVRQAPGKGLEWVAVISY	DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
VH_3-30-3	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYAMH	WVRQAPGKGLEWVAVISY	DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
VH_3-33	QVQLVESGGGVVQPGRSLRLSCAASGFTFS SYGMH	WVRQAPGKGLEWVAVIWY	DGSNKYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
VH_3-35	EVQLVESGGGLVQPGGSLRLSCAASGFTFS NSDMN	WVHQAPGKGLEWVSGVSW	NGSRTHYADSVKG	RFIISRDNSRNTLYLQTNSLRAEDTAVYYCVR		
VH_3-38	EVQLVESGGGLVQPRGSLRLSCAASGFTVS SNEMS	WIRQAPGKGLEWVSSIS	GGSTYYADSRKG	RFTISRDNSKNTLYLQMNNLRAEGTAAYYCARY		
VH_3-43	EVQLVESGGVVVQPGGSLRLSCAASGFTFD DYTMH	WVRQAPGKGLEWVSLISW	DGGSTYYADSVKG	RFTISRDNSKNSLYLQMNSLRTEDTALYYCAKD		
VH_3-48	EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYSMN	WVRQAPGKGLEWVSYISS	SSSTIYYADSVKG	RFTISRDNAKNSLYLQMNSLRAEDTAVYYCAR		
VH_3-49	EVQLVESGGGLVQPGRSLRLSCTASGFTFG DYAMS	WFRQAPGKGLEWVGFIRSKAYGGTTEYTASVKG	RFTISRDGSKSIAYLQMNSLKTEDTAVYYCTR			
VH_3-53	EVQLVESGGGLIQPGGSLRLSCAASGFTVS SNYMS	WVRQAPGKGLEWVSVIY	SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
VH_3-64	EVQLVESGGGLVQPGGSLRLSCAASGFTFS SYAMH	WVRQAPGKGLEYVSAISS	NGGSTYYANSVKG	RFTISRDNSKNTLYLQMGSLRAEDMAVYYCAR		
VH_3-66	EVQLVESGGGLVQPGGSLRLSCAASGFTVS SNYMS	WVRQAPGKGLEWVSVIY	SGGSTYYADSVKG	RFTISRDNSKNTLYLQMNSLRAEDTAVYYCAR		
VH_3-72	EVQLVESGGGLVQPGGSLRLSCAASGFTFS DHYMD	WVRQAPGKGLEWVGRTRNKANSYTTEYAASVKG	RFTISRDDSKNSLYLQMNSLKTEDTAVYYCAR			
VH_3-73	EVQLVESGGGLVQPGGSLKLSCAASGFTFS GSAMH	WVRQASGKGLEWVGRIRSKANSYATAYAASVKG	RFTISRDDSKNTAYLQMNSLKTEDTAVYYCTR			

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VH_3-74    EVQLVESGGGLVQPGGSLRLSCAASGFTFSSYWMH  WVRQAPGKGLVWVSRINS  DGSSTSYADSVKGRFTISRDNAKNTLYLQMNSLRAEDTAVYYCAR
VH_3-d     EVQLVESRGVLVQPGGSLRLSCAASGFTVSSNEMS  WVRQAPGKGLEWVSSIS     GGSTYYADSRKGRFTISRDNSKNTLHLQMNSLRAEDTAVYYCKK
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Figure 1a (continued)

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Chain                FR1          CDR1        FR2        CDR2                  FR3                        CDR3
                      1         2         3           4         5            6         7         8            9         10
Kabat      1234567890123456789012345678901234567890123456789012abc3456789012345678901234567890123456789012abc3456789012345678901234567890abcdefghijk12

VH_4-4     QVQLQESGPGLVKPPGTLSLTCAVSGGSISSSNWWS WVRQPPGKGLEWIGEIY    HSGSTNYNPSLKSRVTISVDKSKNQFSLKLSSVTAADTAVYCCAR
VH_4-28    QVQLQESGPGLVKPSDTLSLTCAVSGYSISSSNWWG WIRQPPGKGLEWIGYIY    YSGSTYYNPSLKSRVTMSVDTSKNQFSLKLSSVTAVDTAVYYCAR
VH_4-30-2  QLQLQESGSGLVKPSQTLSLTCAVSGGSISSGGYSWSWIRQPPGKGLEWIGYIY    HSGSTYYNPSLKSRVTISVDRSKNQFSLKLSSVTAADTAVYYCAR
VH_4-30-4  QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGDYYWSWIRQPPGKGLEWIGYIY    YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-31    QVQLQESGPGLVKPSQTLSLTCTVSGGSISSGGYYWSWIRQHPGKGLEWIGYIY    YSGSTYYNPSLKSLVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-34    QVQLQQWGAGLLKPSETLSLTCAVYGGSFSGYYWS  WIRQPPGKGLEWIGEIN    HSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-39    QLQLQESGPGLVKPSETLSLTCTVSGGSISSSSYYWGWIRQPPGKGLEWIGSIY    YSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-59    QVQLQESGPGLVKPSETLSLTCTVSGGSISSYYWS  WIRQPPGKGLEWIGYIY    YSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-61    QVQLQESGPGLVKPSETLSLTCTVSGGSVSSGSYYWSWIRQPPGKGLEWIGYIY    YSGSTNYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR
VH_4-b     QVQLQESGPGLVKPSETLSLTCAVSGYSISSGYYWG WIRQPPGKGLEWIGSIY    HSGSTYYNPSLKSRVTISVDTSKNQFSLKLSSVTAADTAVYYCAR

VH_5-51    EVQLVQSGAEVKKPGESLKISCKGSGYSFTSYWIG  WVRQMPGKGLEWMGIIYP   GDSDTRYSPSFQGQVTISADKSISTAYLQWSSLKASDTAMYYCAR
VH_5-a     EVQLVQSGAEVKKPGESLRISCKGSGYSFTSYWIS  WVRQMPGKGLEWMGRIDP   SDSYTNYSPSFQGHVTISADKSISTAYLQWSSLKASDTAMYYCAR

VH_6-1     QVQLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWLGRTYYR SKWYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCAR

VH_7-4-1   QVQLVQSGSELKKPGASVKVSCKASGYTFTSYAMN  WVRQAPGQGLEWMGWINT   NTGNPTYAQGFTGRFVFSLDTSVSTAYLQICSLKAEDTAVYYCAR
VH_7-81    QVQLVQSGHEVKQPGASVKVSCKASGYSFTTYGMN  WVPQAPGQGLEWMGWFNT   YTGNPTYAQGFTGRFVFSMDTSASTAYLQISSLKAEDMAMYYCAR
```

Figure 1a. Human Germline VLk Sequences

Chain	FR1	CDR1	FR2	CDR2	FR3	CDR3
Kabat	1 2 3 4 5 6 7 8 9					
	12345678901234567890123456 7abcdef 8901234567890123456789012345 6789012345678901234567890123456789012345 abcdef67					
VLk_1-5	DIQMTQSPSTLSASVGDRVTITC RASQ	SISSWLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS
VLk_1-6	AIQMTQSPSSLSASVGDRVTITC RASQ	GIRNDLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP
VLk_1-8	AIRMTQSPSSFSASTGDRVTITC RASQ	GISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSFP
VLk_1D-8	VIWMTQSPSLLSASTGDRVTISC RMSQ	GISSYLA	WYQQKPGKAPELLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSFP
VLk_1-9	DIQLTQSPSFLSASVGDRVTITC RASQ	GISSYLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP
VLk_1-12	DIQMTQSPSSVSASVGDRVTITC RASQ	GISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP
VLk_1D-12	DIQMTQSPSSVSASVGDRVTITC RASQ	GISSWLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP
VLk_1-13_02	AIQLTQSPSSLSASVGDRVTITC RASQ	GISSALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFNSYP
VLk_1D-13	AIQLTQSPSSLSASVGDRVTITC RASQ	GISSALA	WFQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP
VLk_1-16	DIQMTQSPSSLSASVGDRVTITC RASQ	GISNYLA	WFQQKPGKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP
VLk_1D-16	DIQMTQSPSSLSASVGDRVTITC RASQ	GISSWLA	WYQQKPEKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP
VLk_1-17	DIQMTQSPSSLSASVGDRVTITC RASQ	GIRNDLG	WYQQKPGKAPKRLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP
VLk_1D-17	NIQMTQSPSAMSASVGDRVTITC RARQ	GISNYLA	WFQQKPGKVPKHLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP
VLk_1-27	DIQMTQSPSSLSASVGDRVTITC RASQ	GISNYLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QKYNSAP
VLk_1-33	DIQMTQSPSSLSASVGDRVTITC QASQ	DISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
VLk_1D-33	DIQMTQSPSSLSASVGDRVTITC QASQ	DISNYLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
VLk_1-37	DIQLTQSPSSLSASVGDRVTITC RVSQ	GISSYLN	WYRQKPGKVPKLLIY	SASNLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYG	QRTYNAP
VLk_1D-37	DIQLTQSPSSLSASVGDRVTITC RVSQ	GISSYLN	WYRQKPGKVPKLLIY	SASNLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYG	QRTYNAP
VLk_1-39	DIQMTQSPSSLSASVGDRVTITC RASQ	SISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
VLk_1D-39	DIQMTQSPSSLSASVGDRVTITC RASQ	SISSYLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
VLk_1D-42	DIQMIQSPSFLSASVGDRVSIIC WASE	GISSNLA	WYLQKPGKSPKLFLY	DAKDLHP	GVSSRFSGRGSGTDFTLTIISLKPEDFAAYYC	KQDFSYP
VLk_1D-43	AIRMTQSPFSLSASVGDRVTITC WASQ	GISSYLA	WYQQKPAKAPKLFIY	YASSLQS	GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC	QQYYSTP
VLk_2-24	DIVMTQTPLSSPVTLGQPASISC RSSQSLVHS	DGNTYLS	WLQQRPGQPPRLLIY	KISNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC	MQATQFP
VLk_2D-24	DIVMTQTPLSSPVTLGQPASISF RSSQSLVHS	DGNTYLS	WLQQRPGQPPRLLIY	KVSNRFS	GVPDRFSGSGAGTDFTLKISRVEAEDVGVYYC	TQATQFP
VLk_2-28	DIVMTQSPLSLPVTPGEPASISC RSSQSLLHS	NGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP
VLk_2D-28	DIVMTQSPLSLPVTPGEPASISC RSSQSLLHS	NGYNYLD	WYLQKPGQSPQLLIY	LGSNRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQALQTP
VLk_2D-29	DIVMTQTPLSLSVTPGQPASISC KSSQSLLHS	DGKTYLY	WYLQKPGQPPQLLIY	EVSNRFS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQSIQLP
VLk_2-29_2	DIVMTQTPLSLSVTPGQPASISC KSSQSLLHS	DGKTYLY	WYLQKPGQSPQLLIY	EVSSRFS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGIHLP
VLk_2-30	DVVMTQSPLSLPVTLGQPASISC RSSQSLVYS	DGNTYLN	WFQQRPGQSPRRLIY	KVSNRDS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGTHWP
VLk_2D-30	DVVMTQSPLSLPVTLGQPASISC RSSQSLVYS	DGNTYLN	WFQQRPGQSPRRLIY	KVSNWDS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQGTHWP
VLk_2-40	DIVMTQTPLSLPVTPGEPASISC RSSQSLLDSDDGNTYLD	WYLQKPGQSPQLLIY	TLSYRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQRIEFP	
VLk_2D-40	DIVMTQTPLSLPVTPGEPASISC RSSQSLLDSDDGNTYLD	WYLQKPGQSPQLLIY	TLSYRAS	GVPDRFSGSGSGTDFTLKISRVEAEDVGVYYC	MQRIEFP	

Figure 1b (continued)

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Chain                    FR1             CDR1          FR2     CDR2          FR3                CDR3
                          1         2         3              4         5         6         7         8         9
Kabat      123456789012345678901234567abcdef89012345678901234567890123456789012345678901234567890123456789012345abcdef67

VLk_3-7    EIVMTQSPPTLSLSPGERVTLSCRASQS      VSSSYLTWYQQKPGQAPRLLIYGASTRATSIPARFSGSGSGTDFTLTISSLQPEDFAVYYCQQDHNLP
VLk_3D-7   EIVMTQSPATLSLSPGERATLSCRASQS      VSSSYLSWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTDFTLTISSLQPEDFAVYYCQQDYNLP
VLk_3-11   EIVLTQSPATLSLSPGERATLSCRASQ       SVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGSGTDFTLTISSLEPEDFAVYYCQQRSNWP
VLk_3D-11  EIVLTQSPATLSLSPGERATLSCRASQ       GVSSYLAWYQQKPGQAPRLLIYDASNRATGIPARFSGSGPGTDFTLTISSLEPEDFAVYYCQQRSNWH
VLk_3-15   EIVMTQSPATLSVSPGERATLSCRASQ       SVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWP
VLk_3D-15  EIVMTQSPATLSVSPGERATLSCRASQ       SVSSNLAWYQQKPGQAPRLLIYGASTRATGIPARFSGSGSGTEFTLTISSLQSEDFAVYYCQQYNNWP
VLk_3-20   EIVLTQSPGTLSLSPGERATLSCRASQS      VSSSYLAWYQQKPGQAPRLLIYGASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSP
VLk_3D-20  EIVLTQSPATLSLSPGERATLSCGASQS      VSSSYLAWYQQKPGLAPRLLIYDASSRATGIPDRFSGSGSGTDFTLTISRLEPEDFAVYYCQQYGSSP

VLk_4-1    DIVMTQSPDSLAVSLGERATINCKSSQSVLYSSNNKNYLAWYQQKPGQPPKLLIYWASTRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYSTP

VLk_5-2    ETTLTQSPAFMSATPGDKVNISCKASQ       DIDDDMNWYQQKPGEAAIFIIQEATTLVPGIPPRFSGSGYGTDFTLTINNIESEDAAYYFCLQHDNFP

VLk_6-21   EIVLTQSPDFQSVTPKEKVTITCRASQ       SIGSSLHWYQQKPDQSPKLLIKYASQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSSSLP
VLk_6D-21  EIVLTQSPDFQSVTPKEKVTITCRASQ       SIGSSLHWYQQKPDQSPKLLIKYASQSFSGVPSRFSGSGSGTDFTLTINSLEAEDAATYYCHQSSSLP
VLk_6D-41  DVVMTQSPAFLSVTPGEKVTITCQASE       GIGNYLYWYQQKPDQAPKLLIKYASQSISGVPSRFSGSGSGTDFTFTISSLEAEDAATYYCQQGNKHP
```

Figure 1c

	9 10 678901234567
Kabat	
IGKJ1	WTFGQGTKVEIK
IGKJ2	YTFGQGTKLEIK
IGKJ3	FTFGPGTKVDIK
IGKJ4	LTFGGGTKVEIK
IGKJ5	ITFGQGTRLEIK

	10 11 1234567890123
Kabat	
IGHJ1	AEYFQHWGQGTLVTVSS
IGHJ2	YWYFDLWGRGTLVTVSS
IGHJ3	AFDVWGQGTMVTVSS
IGHJ4	YFDYWGQGTLVTVSS
IGHJ5	NWFDSWGQGTLVTVSS
IGHJ6	YYYYYGMDVWGQGTTVTVSS

Figure 2a

QVQLQQSGPELVKPGASLKLSCTASGFNIK
QVQLVQSGAEVKKPGASVKVSCKASGYTFT

IDstring(i=15) = 6

Figure 2b

VH_m4D5	QVQLQQSGPELVKPGASLKLSCTASGFNIK	
VH_1-2	QVQLVQSGAEVKKPGASVKVSCKASGYTFT	IDepitope(i=15) = 6
VH_2-5	QITLKESGPTLVKPTQTLTLTCTFSGFSLS	IDepitope(i=15) = 4
VH_3-7	EVQLVESGGGLVQPGGSLRLSCAASGFTFS	IDepitope(i=15) = 6
VH_4-4	QVQLQESGPGLVKPPGTLSLTCAVSGGSIS	IDepitope(i=15) = 3
VH_5-51	EVQLVQSGAEVKKPGESLKISCKGSGYSFT	IDepitope(i=15) = 6
VH_6-1	QVQLQQSGPGLVKPSQTLSLTCAISGDSVS	IDepitope(i=15) = 3
VH_7-4-1	QVQLVQSGSELKKPGASVKVSCKASGYTFT	IDepitope(i=15) = 6

IDmax(i=15) = 6

Figure 2c

VH_m4D5 QVQLQQSGPELVKPGASLKLSCTASGFNIK
VH_1-2 QVQLVQSGAEVKKPGASVKVSCKASGYTFT
VH_2-5 QITLKESGPTLVKPTQTLTLTCTFSGFSLS
VH_3-7 EVQLVESGGGLVQPGGSLRLSCAASGFTFS
VH_4-4 QVQLQESGPGLVKPPGTLSLTCAVSGGSIS
VH_5-51 EVQLVQSGAEVKKPGESLKISCKGSGYSFT
VH_6-1 QVQLQQSGPGLVKPSQTLSLTCAISGDSVS
VH_7-4-1 QVQLVQSGSELKKPGASVKVSCKASGYTFT

ID(i=1)max = 9 ID(i=12)max = 6
ID(i=2)max = 8 ID(i=13)max = 7
ID(i=3)max = 8 ID(i=14)max = 7
ID(i=4)max = 8 ID(i=15)max = 6
ID(i=5)max = 8 ID(i=16)max = 6
ID(i=6)max = 8 ID(i=17)max = 7
ID(i=7)max = 7 ID(i=18)max = 7
ID(i=8)max = 7 ID(i=19)max = 7
ID(i=9)max = 7 ID(i=20)max = 7
ID(i=10)max = 7 ID(i=21)max = 6
ID(i=11)max = 7 ID(i=22)max = 5

HSC(s) = 78.3

Figure 3. L0 AC10 VL

Figure 3a

DIVLTQSPASLAVSLGQRATISCKASQSVDFDGDSYMNWYQQKPGQPPKVLIYAASNLESG
IPARFSGSGSGTDFTLNIHPVEEEDAATYYCQQSNEDPWTFGGGTKLEIK

Figure 3b

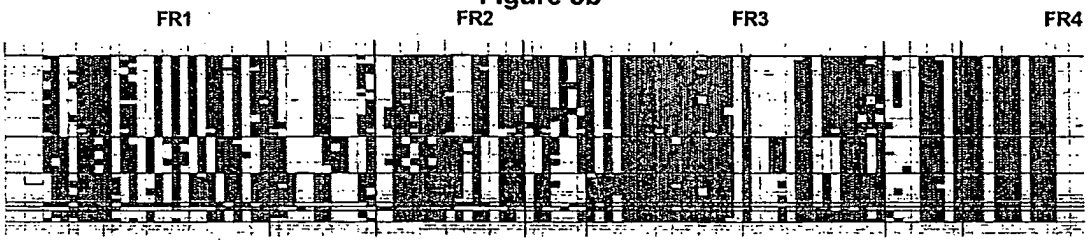

Figure 3c

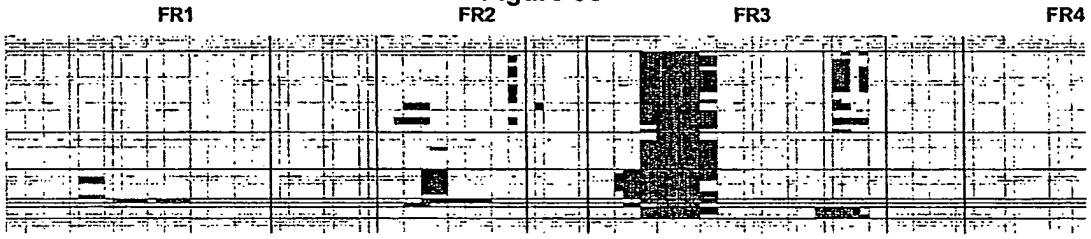

Figure 3d

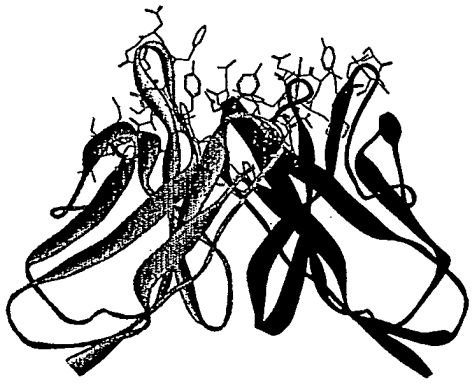

Figure 4. H0 AC10 VH

Figure 4a

QIQLQQSGPEVVKPGASVKISCKASGYTFTDYYITWVKQKPGQGLEWIGWIYPGSGNTKYN
EKFKGKATLTVDTSSSTAFMQLSSLTSEDTAVYFCANYGNYWFAYWGQGTQVTVSA

Figure 4b

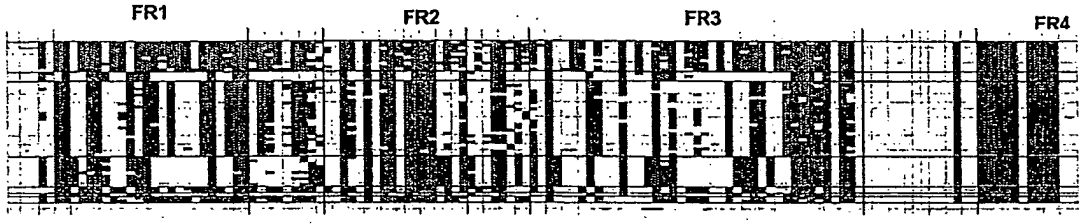

Figure 4c

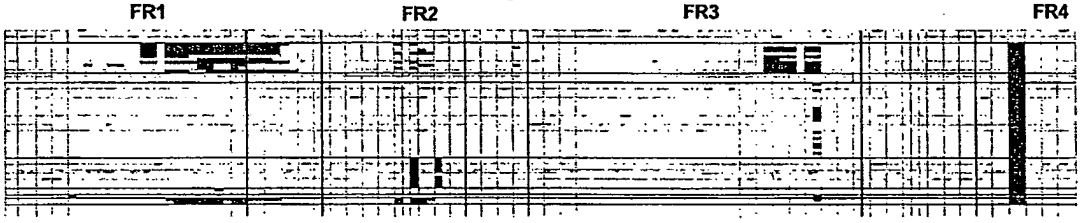

Figure 4d

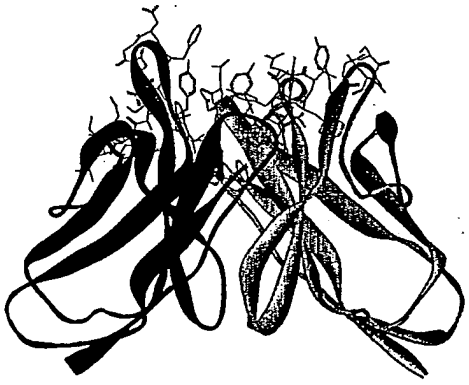

Figure 5. CDR grafted AC10 VL

Figure 5a

DIVMTQSPDSLAVSLGERATINCKSSQSVDFDGDSYLAWYQQKPGQPPKLLIYAASNLESG
VPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQSNEDPWTFGGGTKLEIK

Figure 5b

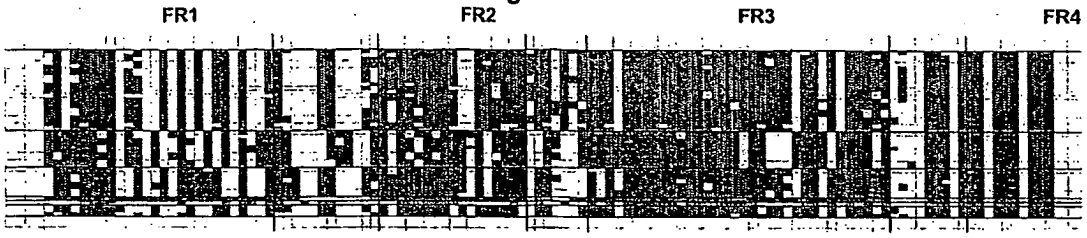

Figure 5c

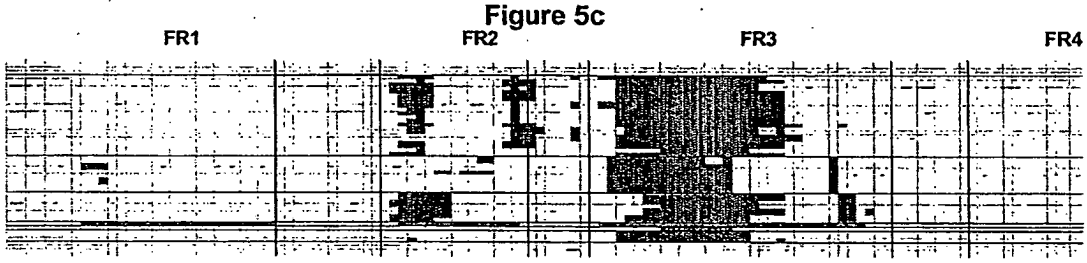

Figure 5d

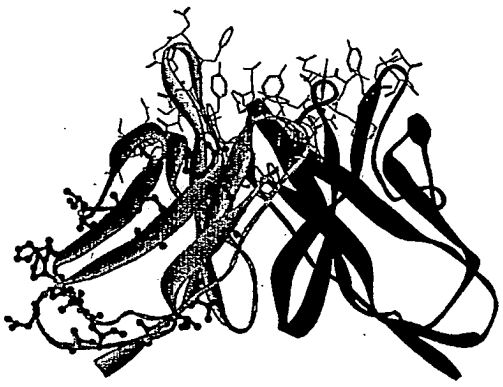

Figure 6. CDR grafted AC10 VH

Figure 6a

QVQLVQSGAEVKKPGASVKVSCKASGYTFTDYYMHWVRQAPGQRLEWMGWIYPGSGNT
KYSQKFQGRVTITRDTSASTAYMELSSLRSEDTAVYYCARYGNYWFAYWGQGTLVTVSS

Figure 6b

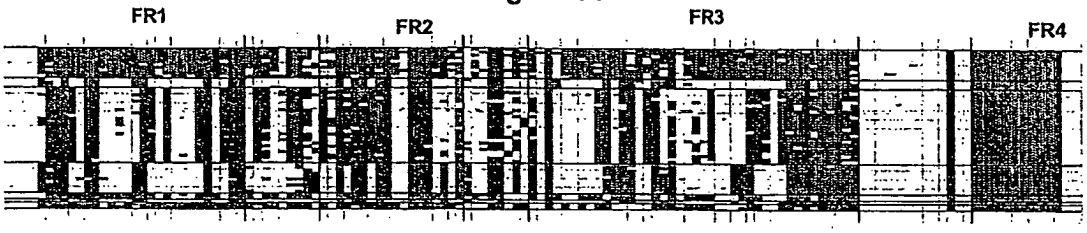

Figure 6c

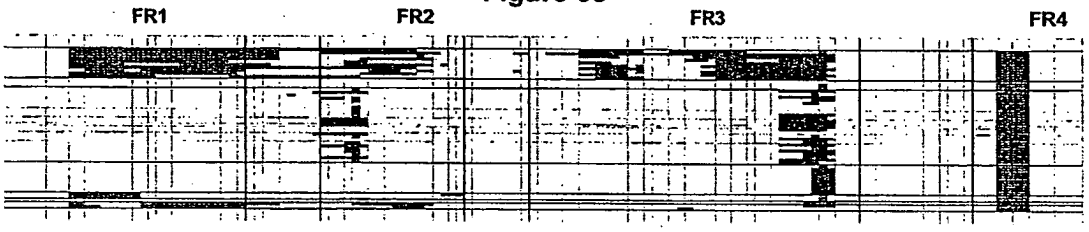

Figure 6d

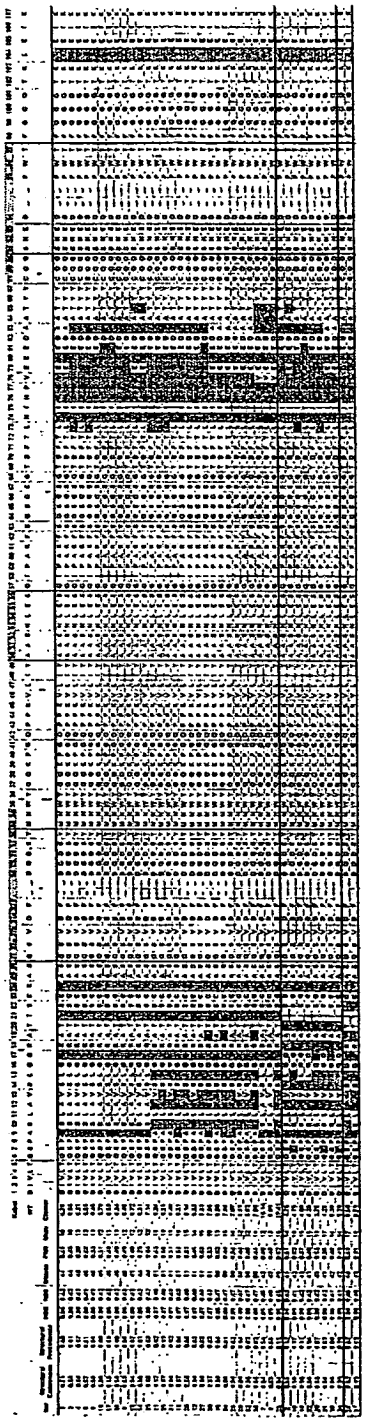

Figure 7a. AC10 VL HEC Calculation 1

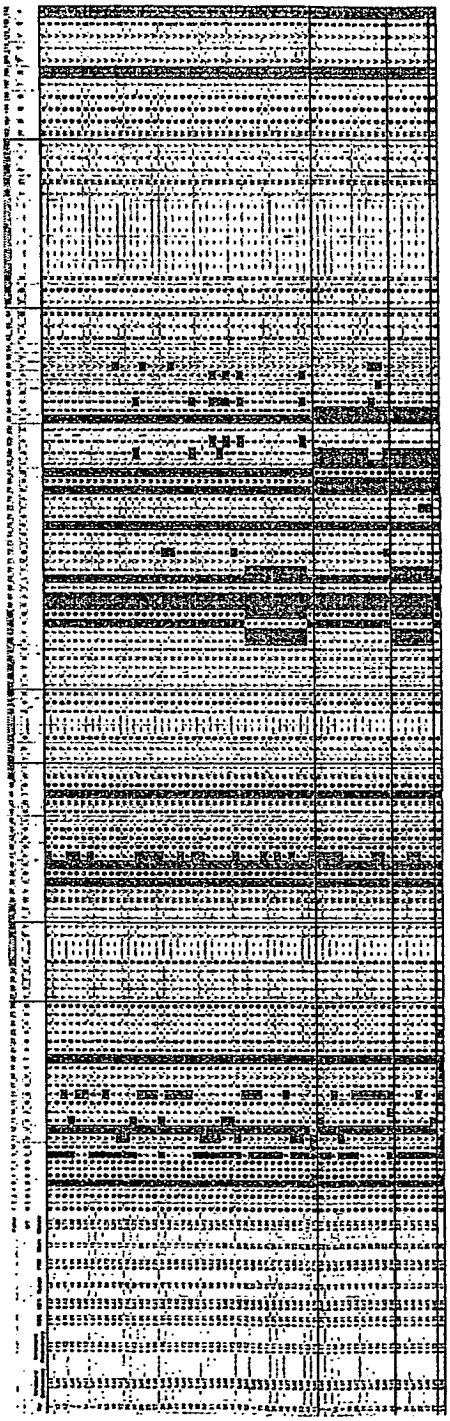

Figure 7b. AC10 VH HEC Calculation 1

Figure 8. L1 AC10 VL

Figure 8a

DIVLTQSPATLSLSPGERATLSCRASQSVDFDGDSYMNWYQQKPGQPPKVLIYAASNLES
GIPARFSGSGSGTDFTLTISSLQPEDFATYYCQQSNEDPWTFGGGTKVEIK

Figure 8b

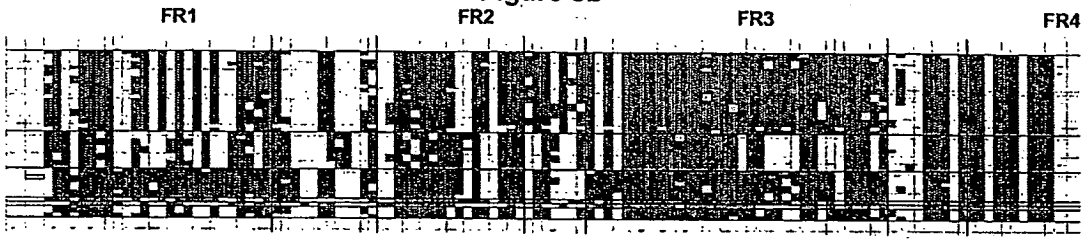

Figure 8c

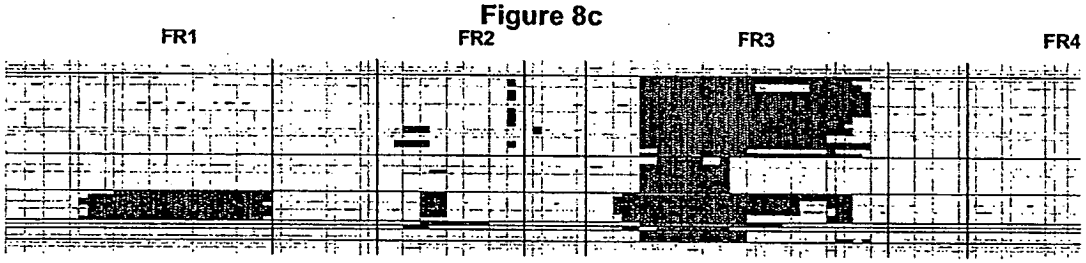

Figure 8d

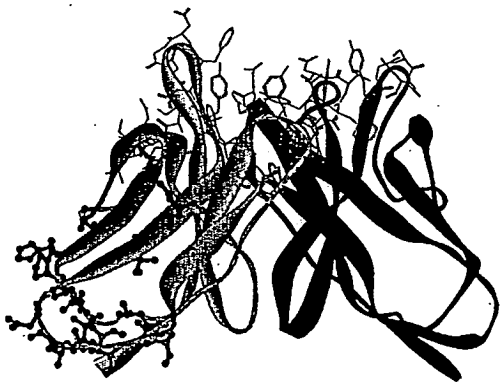

Figure 9. L2 AC10 VL

Figure 9a

DIVLTQSP**S**SL**SA**SVG**D**R**V**TI**T**C**R**ASQSVDFDGDSYMNWYQQKPGQPPKVLIYAASNLESG
IPARFSGSGSGTDFTL**TISSLQP**EDFATYYCQQSNEDPWTFGGGTK**V**EIK

Figure 9b

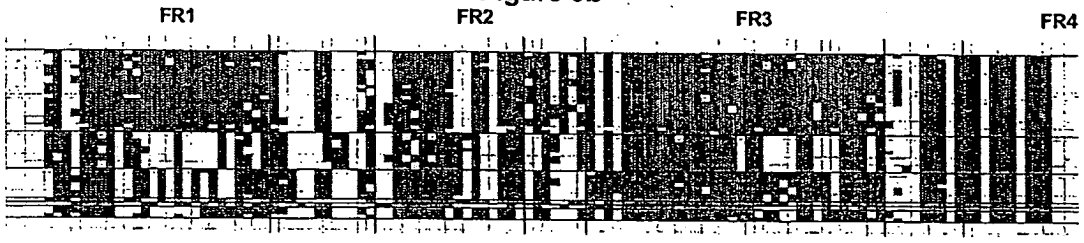

Figure 9c

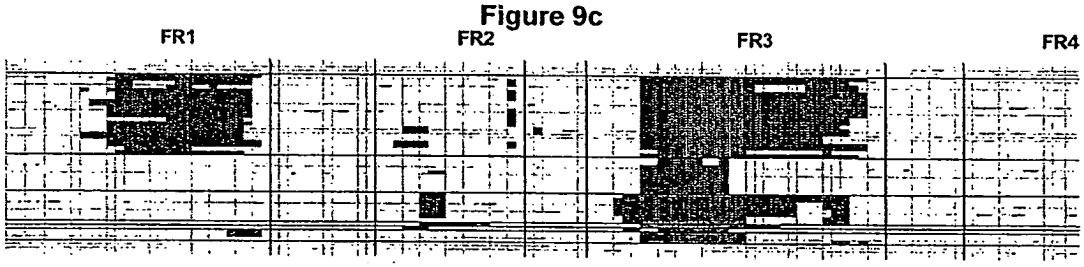

Figure 9d

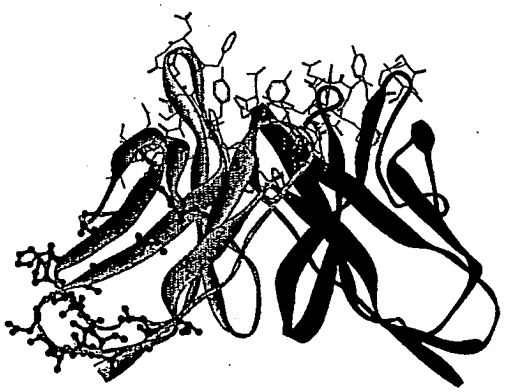

Figure 10. L3 AC10 VL

Figure 10a

DIVLTQSPDSLAVSLGERATINCKASQSVDFDGDSYMNWYQQKPGQPPKVLIYAASNLESG
IPARFSGSGSGTDFTLTINSLEAEDAATYYCQQSNEDPWTFGGGTKVEIK

Figure 10b

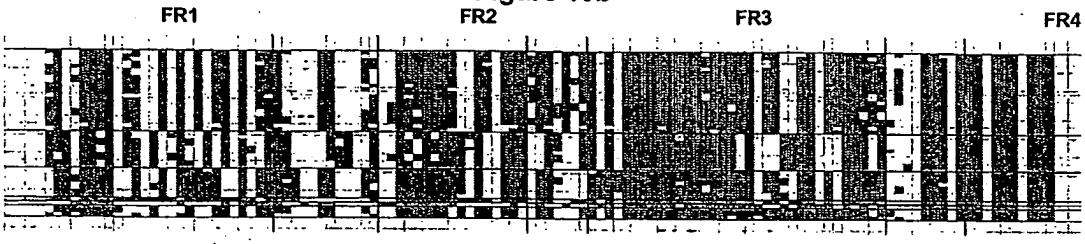

Figure 10c

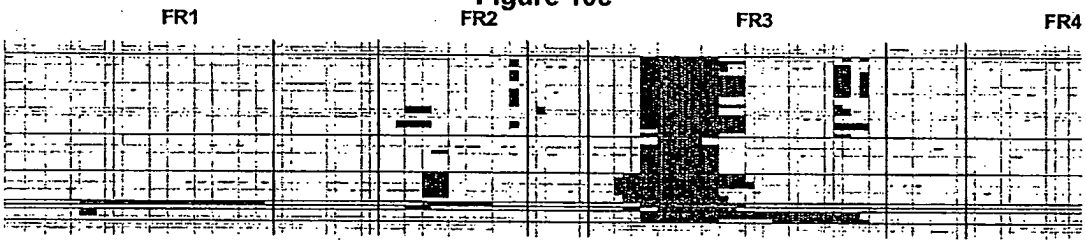

Figure 10d

Figure 11. H1 AC10 VH

Figure 11a

QIQLVQSGPEVKKPGASVKVSCKASGYTFTDYYITWVRQAPGQGLEWMGWIYPGSGNTK
YNEKFQGRVTITVDTSASTAYMELSSLRSEDTAVYFCANYGNYWFAYWGQGTLVTVSS

Figure 11b

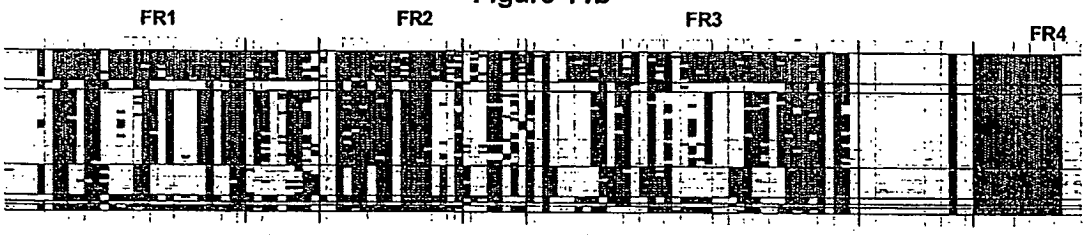

Figure 11c

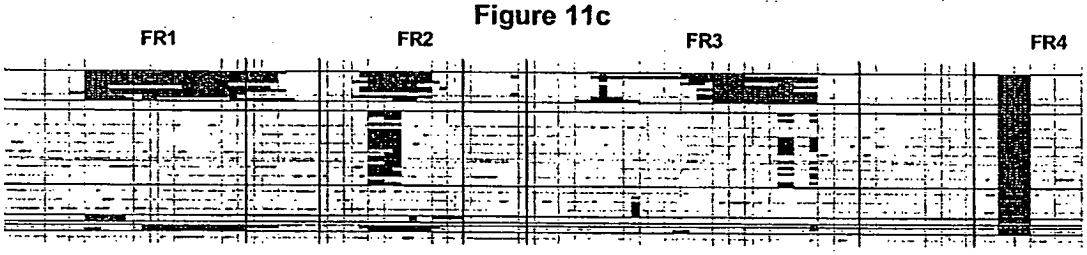

Figure 11d

Figure 12. H2 AC10 VH

Figure 12a

QIQLVESGGGLVKPGGSLRLSCAASGYTFTDYYITWVRQAPGQGLEWMGWIYPGSGNTK
YNEKFQGRVTMTVDTSTSTAYMELSSLRSEDTAVYFCANYGNYWFAYWGQGTLVTVSS

Figure 12b

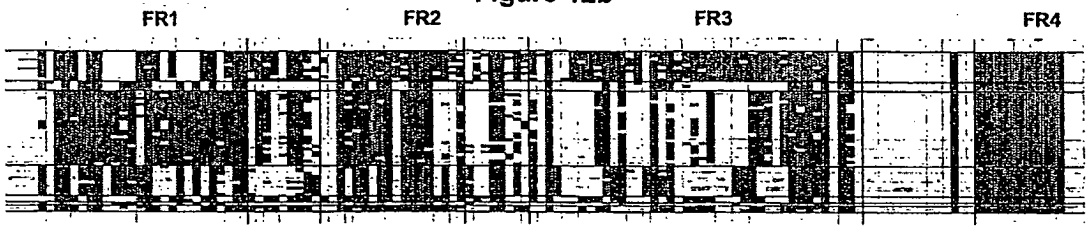

Figure 12c

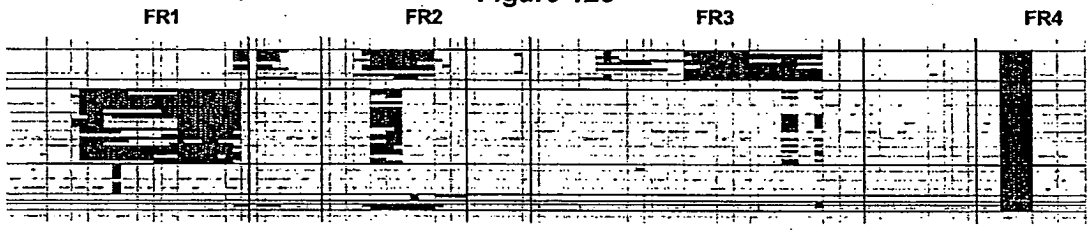

Figure 12d

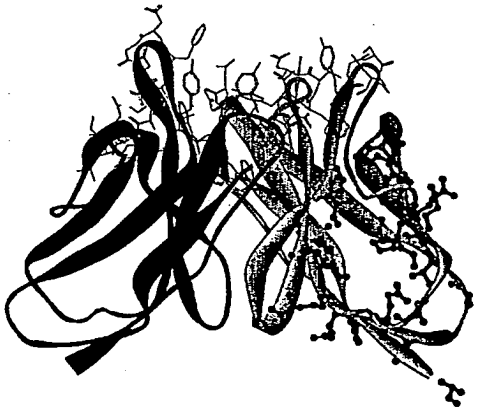

Figure 13. H3 AC10 VH

Figure 13a

QIQLVQSGPEVKKPGASVKVSCKASGYTFTDYYITWVRQAPGQGLEWMGWIYPGSGNTK
YNEKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYFCANYGNYWFAYWGQGTLVTVSS

Figure 13b

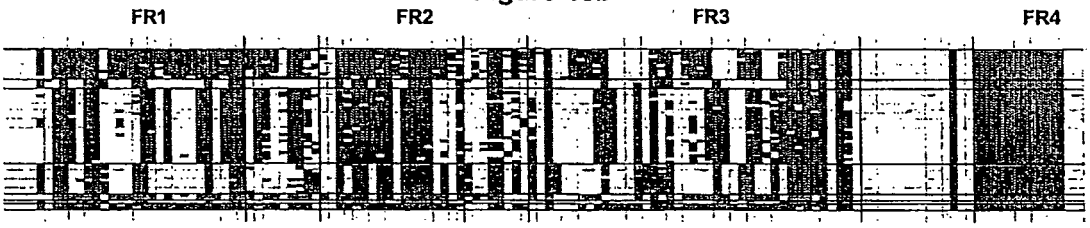

Figure 13c

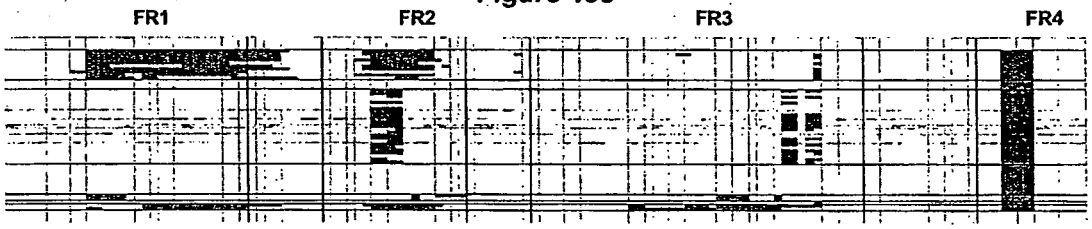

Figure 13d

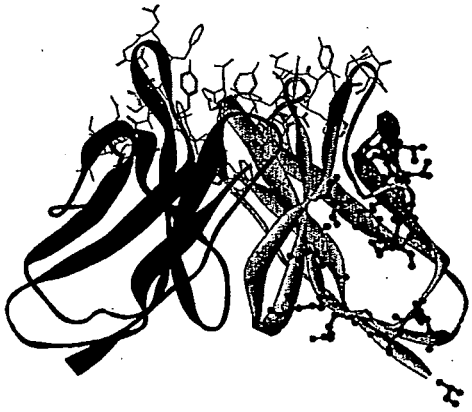

Figure 14a

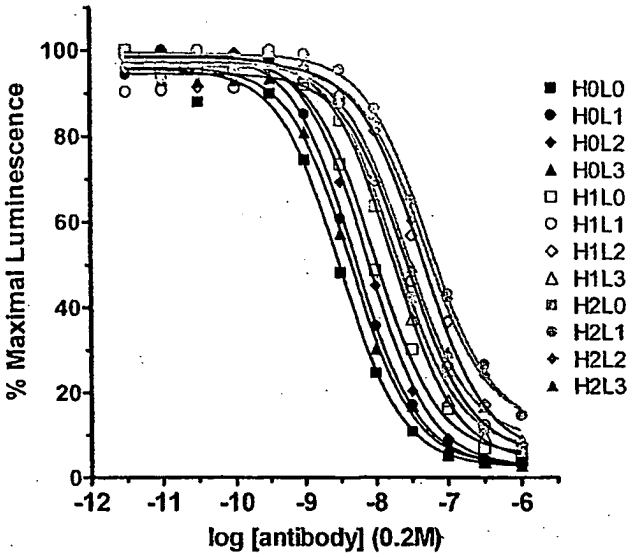

Figure 14b

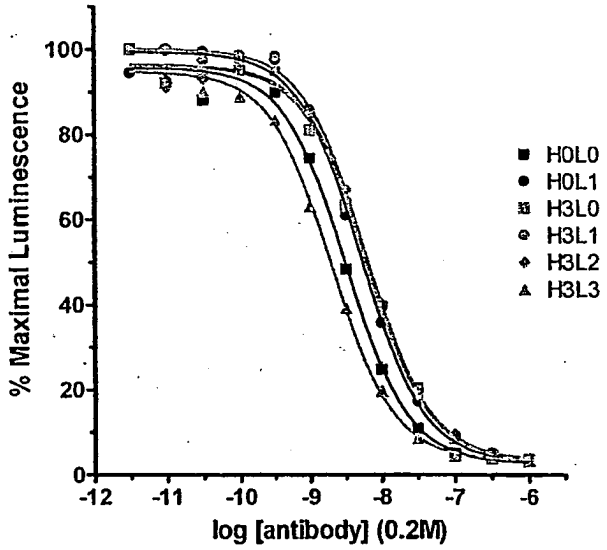

Figure 15

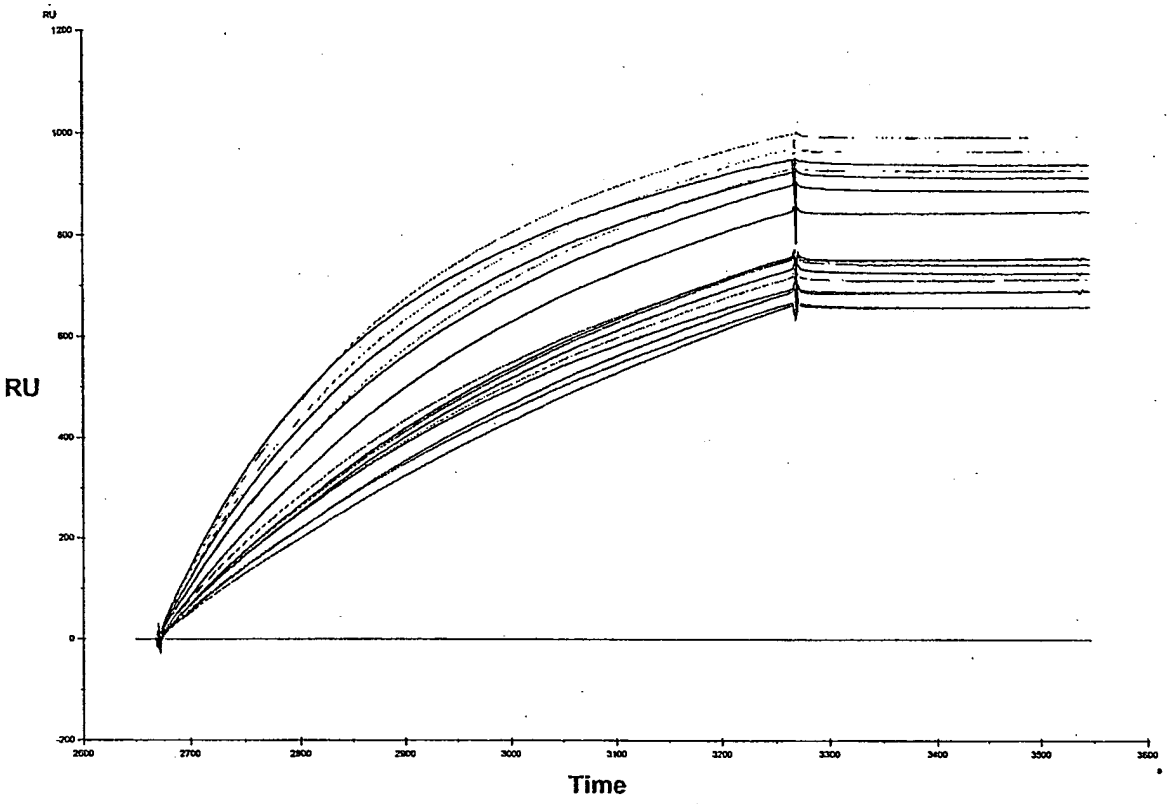
RU
1200
1000
800
600
400
200
0
-200
2600
2700
2800
2900
3000
3100
3200
3300
3400
3500
3600
Time

Figure 16a

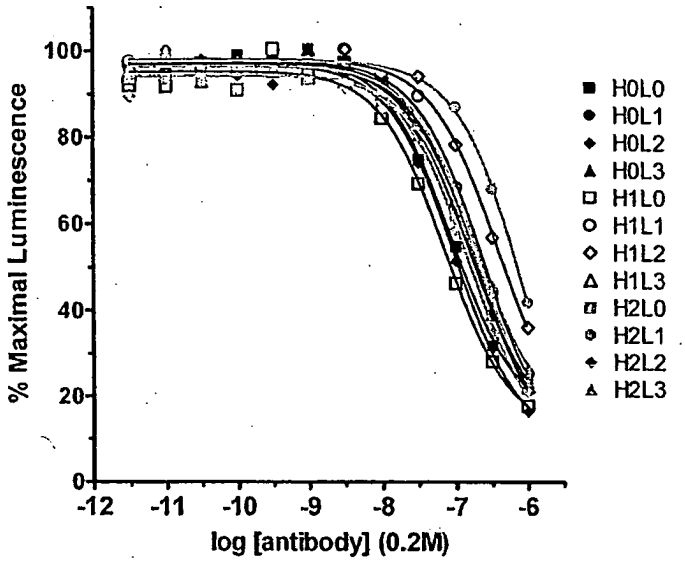
% Maximal Luminescence
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log [antibody] (0.2M)
H0L0
H0L1
H0L2
H0L3
H1L0
H1L1
H1L2
H1L3
H2L0
H2L1
H2L2
H2L3

Figure 16b

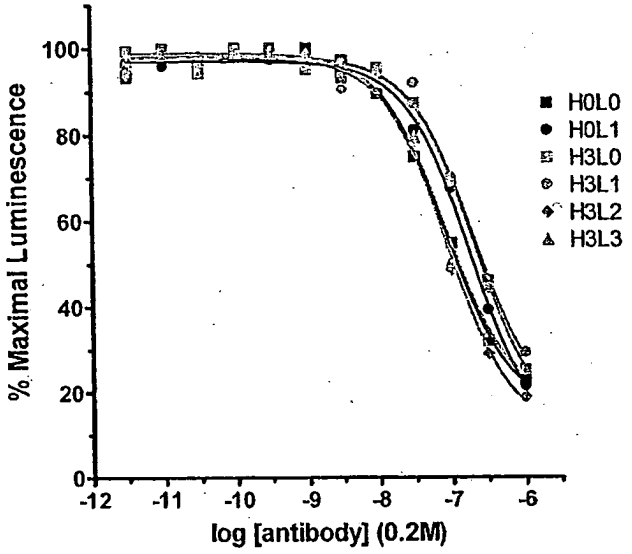
% Maximal Luminescence
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log [antibody] (0.2M)
H0L0
H0L1
H3L0
H3L1
H3L2
H3L3

Figure 17a

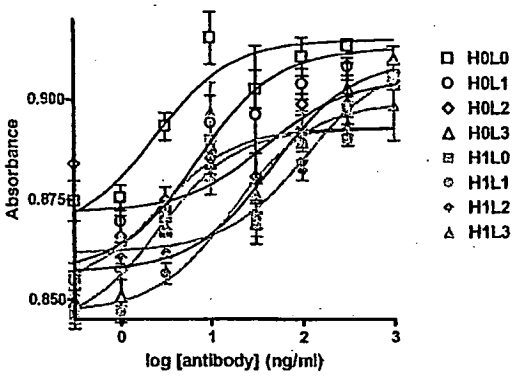

Figure 17b

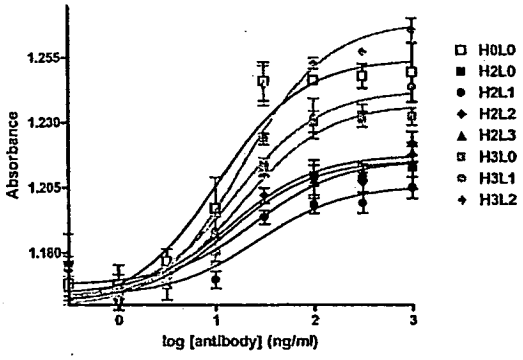

Figure 17c

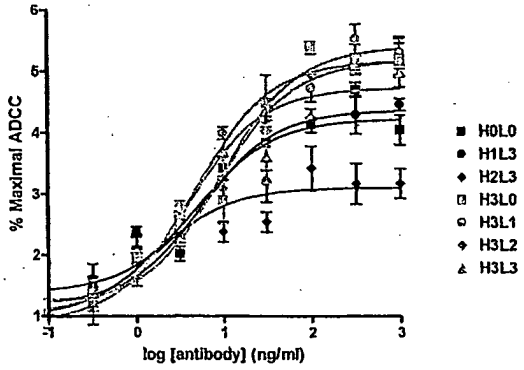
% Maximal ADCC
log [antibody] (ng/ml)
H0L0
H1L3
H2L3
H3L0
H3L1
H3L2
H3L3

Figure 18a

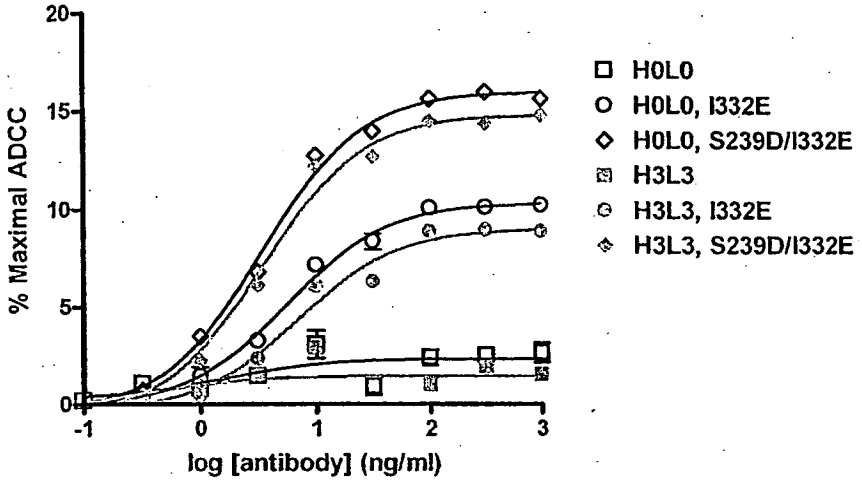
20
15
10
5
0
% Maximal ADCC
-1
0
1
2
3
log [antibody] (ng/ml)
H0L0
H0L0, I332E
H0L0, S239D/I332E
H3L3
H3L3, I332E
H3L3, S239D/I332E

Figure 18b

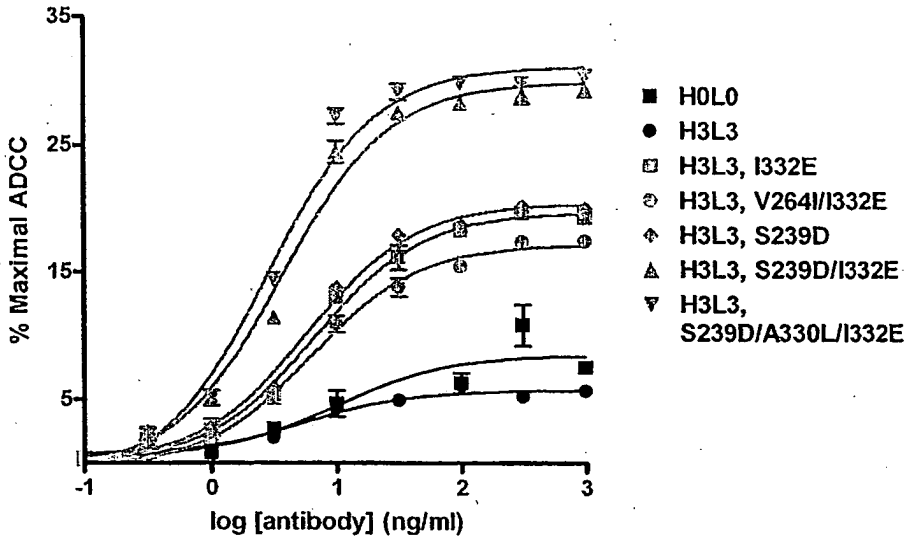
35
25
15
5
% Maximal ADCC
-1
0
1
2
3
log [antibody] (ng/ml)
H0L0
H3L3
H3L3, I332E
H3L3, V264I/I332E
H3L3, S239D
H3L3, S239D/I332E
H3L3, S239D/A330L/I332E

Figure 19

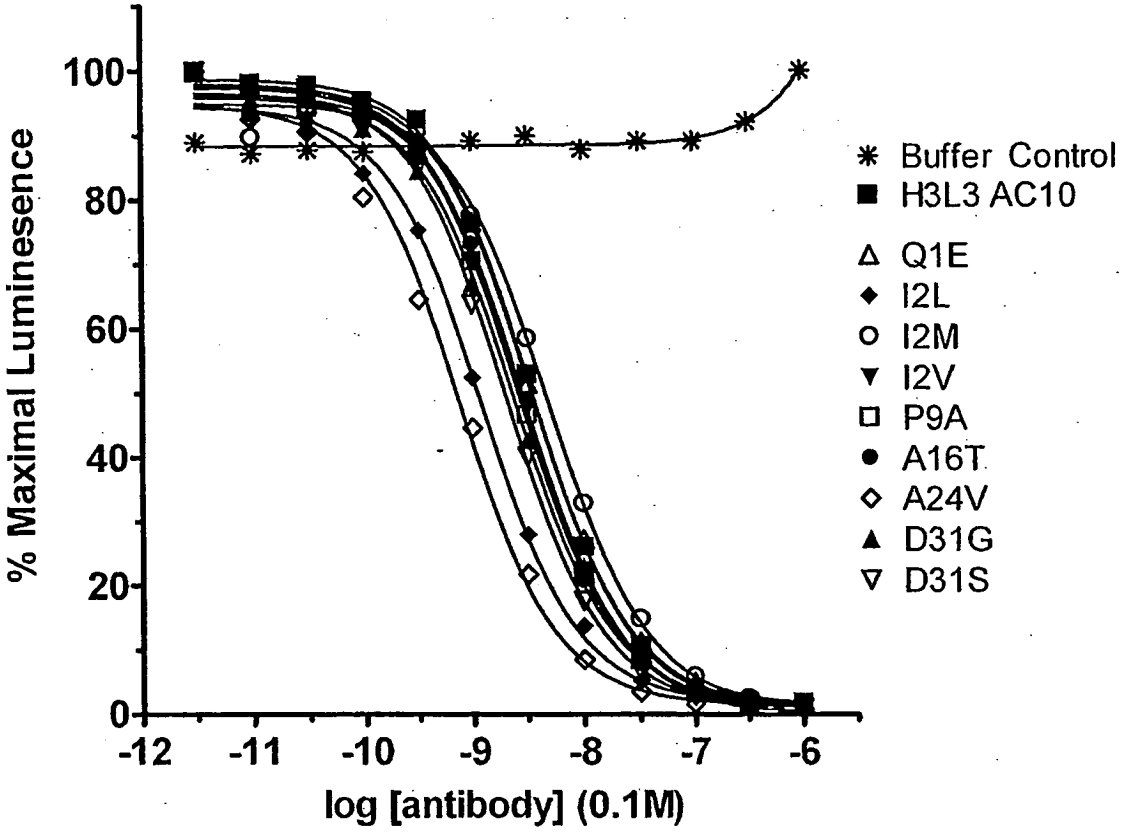
% Maximal Luminesence
100
80
60
40
20
0
-12
-11
-10
-9
-8
-7
-6
log [antibody] (0.1M)
Buffer Control
H3L3 AC10
Q1E
I2L
I2M
I2V
P9A
A16T
A24V
D31G
D31S

Figure 20. L3.71 AC10 VL

Figure 20a

EIVLTQSPDSLAVSLGERATINCKASQSVDFDGDSYLNWYQQKPGQPPKVLIYAASTLQSG
VPSRFSGSGSGTDFTLTINSLEAEDAATYYCQQSNEDPWTFGGGTKVEIK

Figure 20b

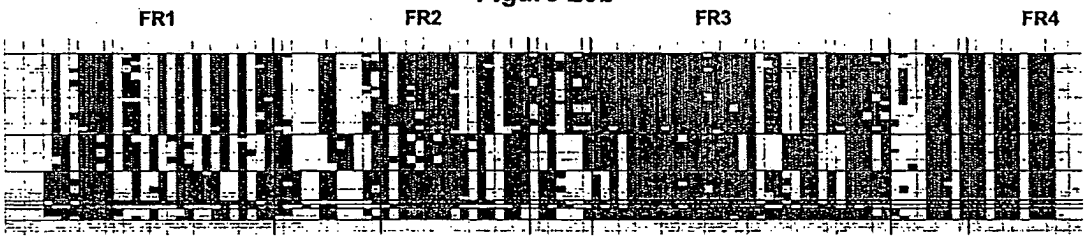

Figure 20c

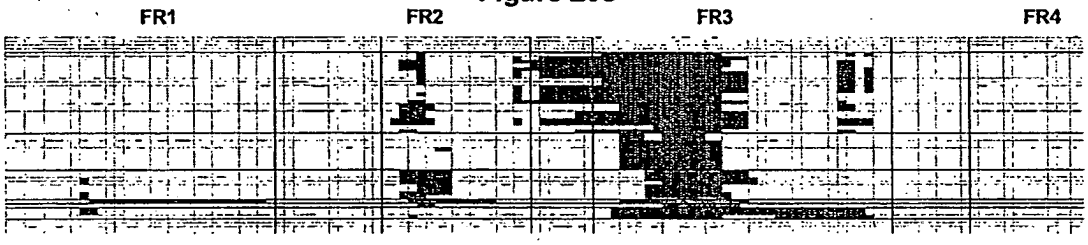

Figure 20d

Figure 21. L3.72 AC10 VL

Figure 21a

AIVLTQSPDSLAVSLGERATINCKASQSVDFDGDSYLNWYQQKPGQPPKVLIYAASTLETG
VPSRFSGSGSGTDFTLTINSLEAEDAATYYCQQSNEDPWTFGGGTKVEIK

Figure 21b

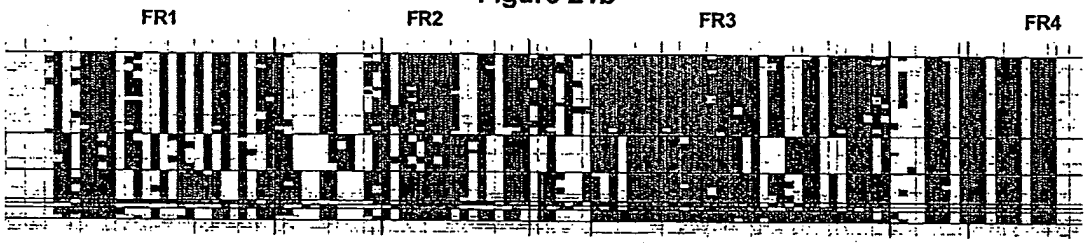

Figure 21c

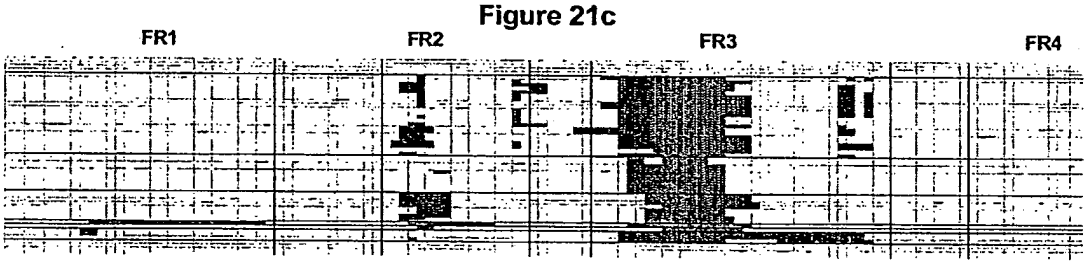

Figure 21d

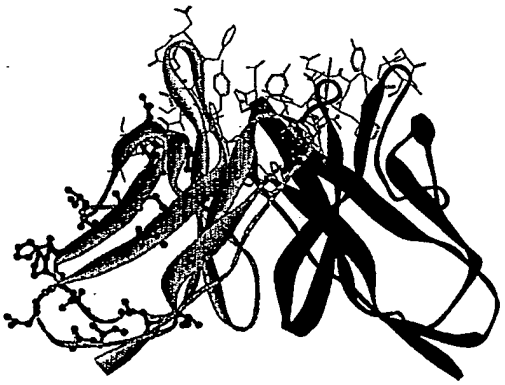

Figure 22. H3.68 AC10 VH

Figure 22a

QLQLVQSGPEVKKPGASVKVSCKVSGYTFTDYYITWVRQAPGQALEWMGWIYPGSGNTK
YNEKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYFCANYGNYWFAYWGQGTLVTVSS

Figure 22b

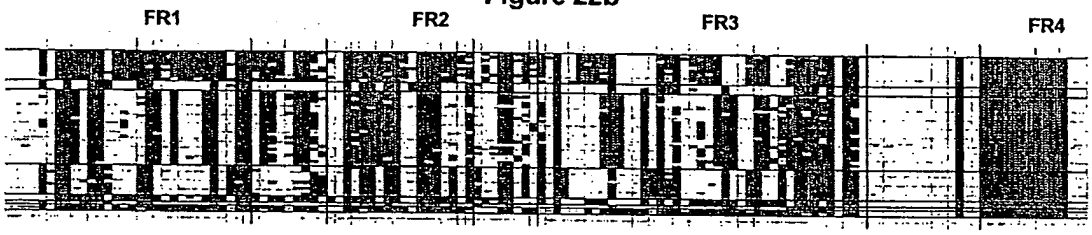

Figure 22c

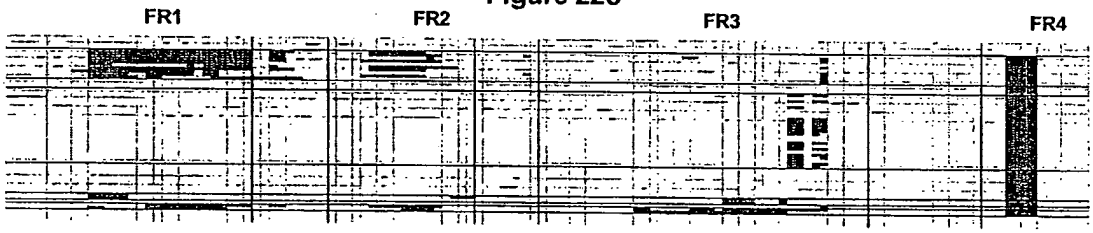

Figure 22d

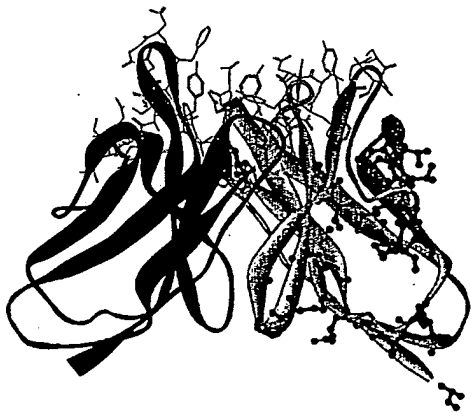

Figure 23. H3.69 AC10 VH

Figure 23a

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTDYYITWVRQAPGQALEWMGWIYPGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSS

Figure 23b

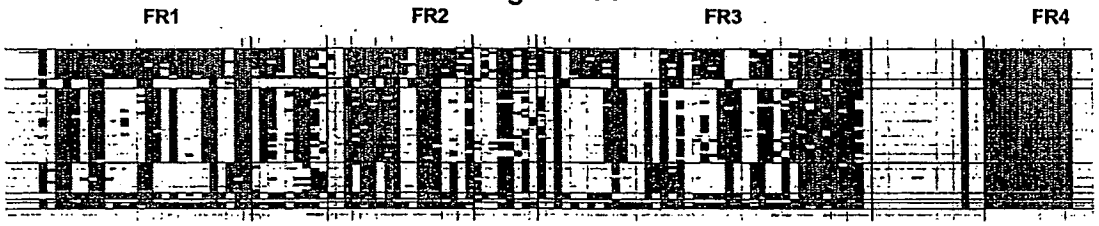

Figure 23c

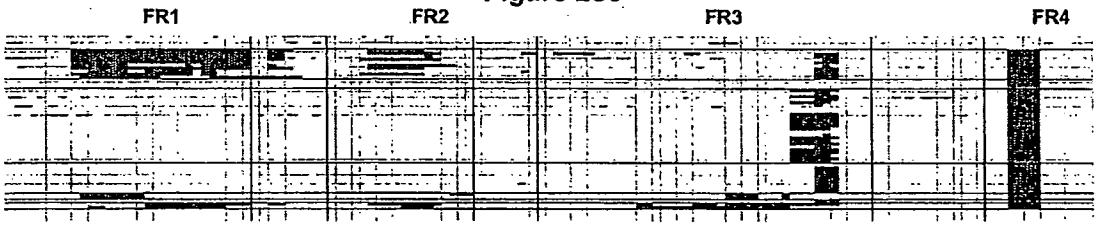

Figure 23d

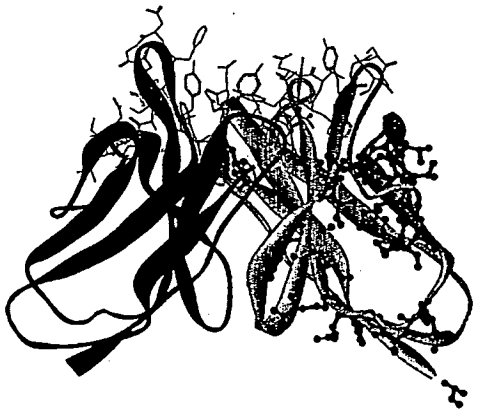

Figure 24. H3.70 AC10 VH

Figure 24a

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTSYYISWVRQAPGQALEWMGWIYAGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSS

Figure 24b

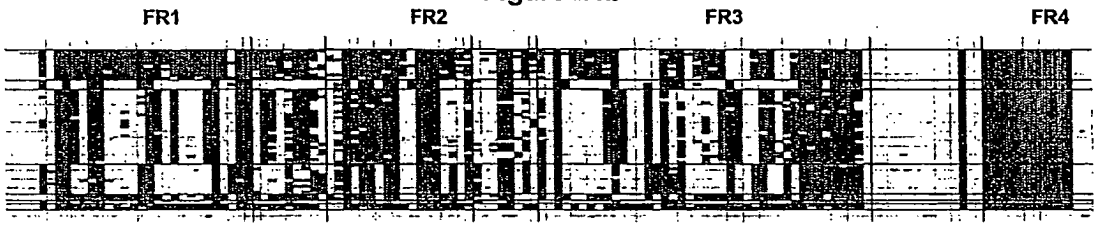

Figure 24c

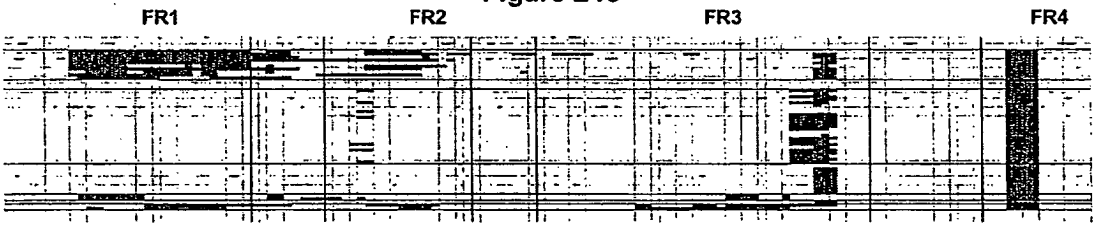

Figure 24d

Figure 25. Optimized Anti-CD30 IgG1 Antibodies

Figure 25a. Anti-CD30 Light Chain

EIVLTQSPDSLAVSLGERATINCKASQSVDFDGDSYLNWYQQKPGQPPKVLIYAASTLQSG
VPSRFSGSGSGTDFTLTINSLEAEDAATYYCQQSNEDPWTFGGGTKVEIKRTVAAPSVFIFP
PSDEQLKSGTASVVCLLNNFYPREAKVQWKVDNALQSGNSQESVTEQDSKDSTYSLSSTL
TLSKADYEKHKVYACEVTHQGLSSPVTKSFNRGEC

Figure 25b. Anti-CD30 IgG1 Heavy Chain Comprising Possible Fc Variants

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTSYYISWVRQAPGQALEWMGWIYAGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSSAS
TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGPX_1VF
LFPPKPKDTLMISRTPEVTCVVX_2DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPX_3PX_4EKTISKAKGQPREPQVYTLPPSRDELTKN
QVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGN
VFSCSVMHEALHNHYTQKSLSLSPGK

Position	EU Index Position	WT	Possible Substitutions
X_1	239	S	D, E, N, Q, T
X_2	264	V	I, T, Y
X_3	330	A	Y, L, I
X_4	332	I	D, E, N, Q

Figure 25c. Anti-CD30 Fc Variant IgG1 Heavy Chain

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTSYYISWVRQAPGQALEWMGWIYAGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSSAS
TKGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDKKAEPKSCDKTHTCPPCPAPELLGGPDVF
LFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR
VVSVLTVLHQDWLNGKEYKCKVSNKALPLPEEKTISKAKGQPREPQVYTLPPSRDELTKNQ
VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV
FSCSVMHEALHNHYTQKSLSLSPGK

Figure 25d. Anti-CD30 IgG2 Heavy Chain Comprising Possible Fc Variants

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTSYYISWVRQAPGQALEWMGWIYAGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSSAS
TKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAP$Z_1Z_2Z_3Z_4$GPX_1VF
LFPPKPKDTLMISRTPEVTCVVX_2DVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFR
VVSVLTVVHQDWLNGKEYKCKVSNKZ_5LPX_3PX_4EKTISKTKGQPREPQVYTLPPSREEMTK
NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQG
NVFSCSVMHEALHNHYTQKSLSLSPGK

Position	EU Index Position	WT	Possible Substitutions
X_1	239	S	D, E, N, Q, T
X_2	264	V	I, T, Y
X_3	330	A	Y, L, I
X_4	332	I	D, E, N, Q
Z_1	233	P	E
Z_2	234	V	L
Z_3	235	A	L
Z_4	236	-	G
Z_5	327	G	A

Figure 25e. Anti-CD30 Fc Variant IgG2 Heavy Chain

QLQLVQSGAEVKKPGASVKVSCKVSGYTFTSYYISWVRQAPGQALEWMGWIYAGSGNTK
YSQKFQGRFVFSVDTSASTAYLQISSLKAEDTAVYYCANYGNYWFAYWGQGTLVTVSSAS
TKGPSVFPLAPCSRSTSESTAALGCLVKDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLY
SLSSVVTVPSSNFGTQTYTCNVDHKPSNTKVDKTVERKCCVECPPCPAP**ELLG**GP**D**VFLFP
PKPKDTLMISRTPEVTCVV**V**DVSHEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTFRVVS
VLTVVHQDWLNGKEYKCKVSNK**GLPLPE**EKTISKTKGQPREPQVYTLPPSREEMTKNQVS
LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPMLDSDGSFFLYSKLTVDKSRWQQGNVFS
CSVMHEALHNHYTQKSLSLSPGK

Figure 26. C225 WT VL

Figure 26a

DILLTQSPVILSVSPGERVSFSCRASQSIGTNIHWYQQRTNGSPRLLIKYASESISGIPSRFSG
SGSGTDFTLSINSVESEDIADYYCQQNNNWPTTFGAGTKLELK

Figure 26b

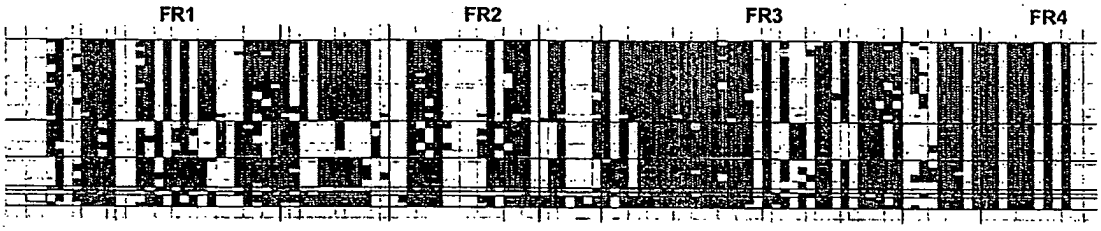

Figure 26c

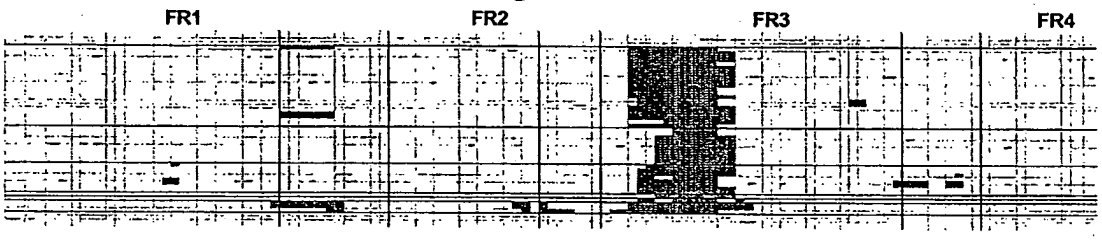

Figure 26d

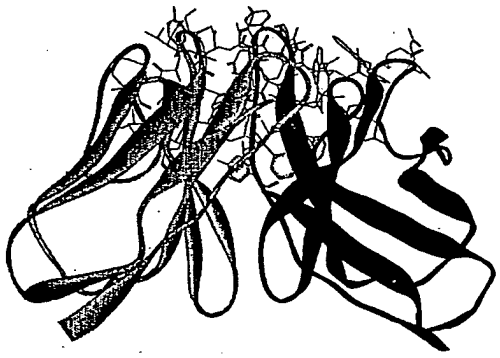

Figure 27. WT C225 VH

Figure 27a

QVQLKQSGPGLVQPSQSLSITCTVSGFSLTNYGVHWVRQSPGKGLEWLGVIWSGGNTDY
NTPFTSRLSINKDNSKSQVFFKMNSLQSNDTAIYYCARALTYYDYEFAYWGQGTLVTVSA

Figure 27b

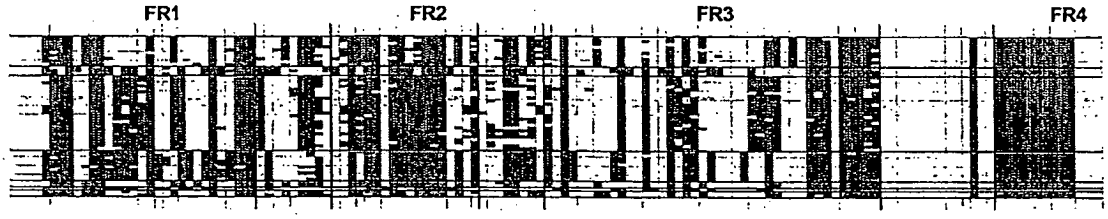

Figure 27c

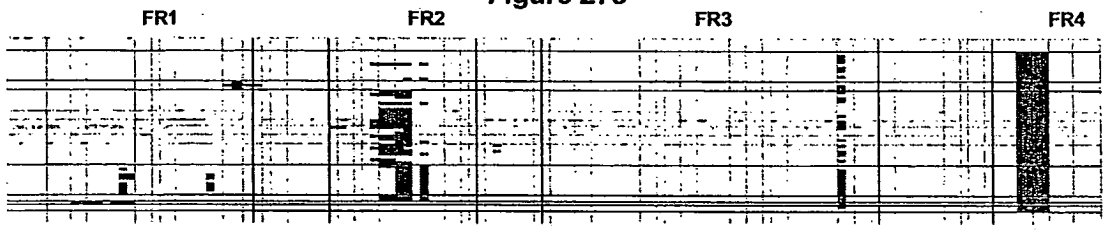

Figure 27d

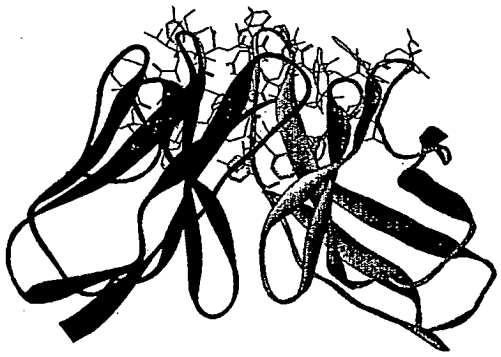

Figure 28. CDR grafted C225 VL

Figure 28a

EIVLTQSPDFQSVTPKEKVTITCRASQSIGTNLHWYQQKPDQSPKLLIKYASESISGVPSRF
SGSGSGTDFTLTINSLEAEDAATYYCHQNNNWPTTFGAGTKLEIK

Figure 28b

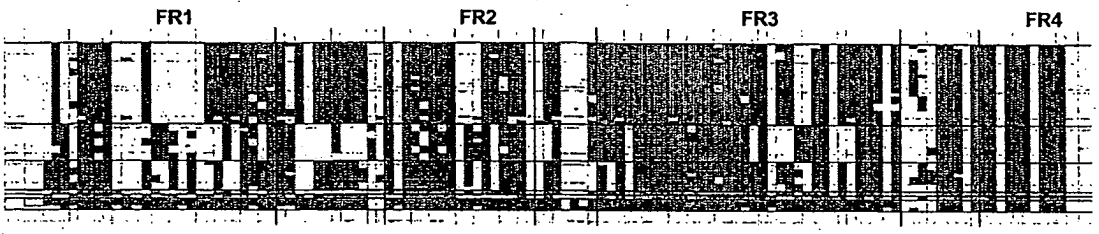

Figure 28c

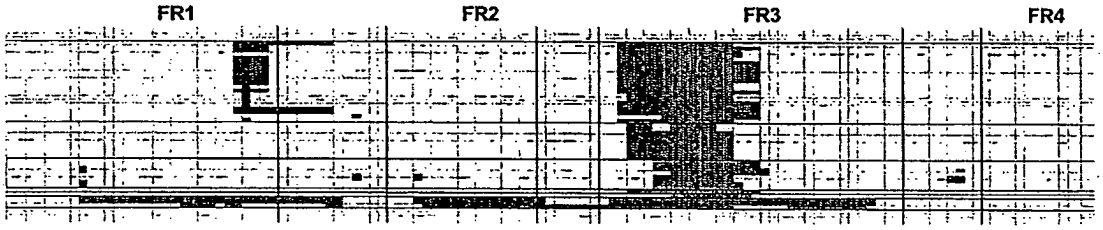

Figure 28d

Figure 29. CDR grafted C225 VH

Figure 29a

QVQL**QE**SGPGLV**K**PSQ**T**LS**L**TCTVSGFSLTNYG**WS**WIRQ**P**PGKGLEWIG**Y**IWSGGNT**Y**YN
PSLKSR**VTI**SVDTSK**NQFSLKLSSVTAA**DTAVYYCARALTYYDYEFAYWGQGTLVTVS**S**

Figure 29b

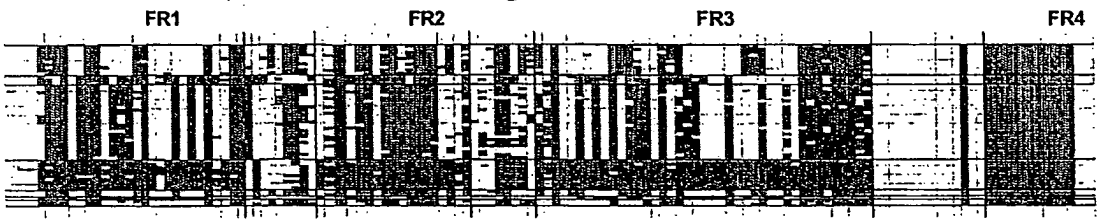

Figure 29c

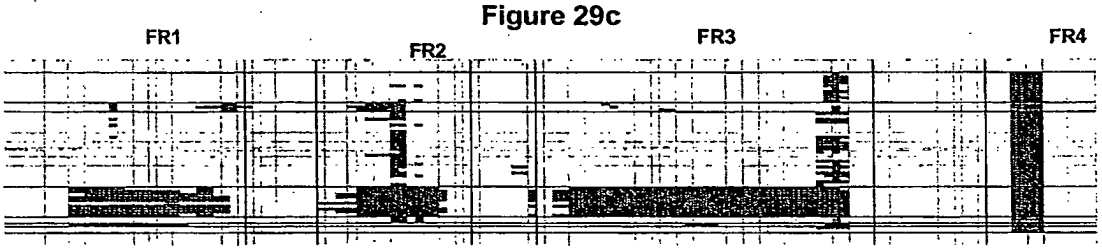

Figure 29d

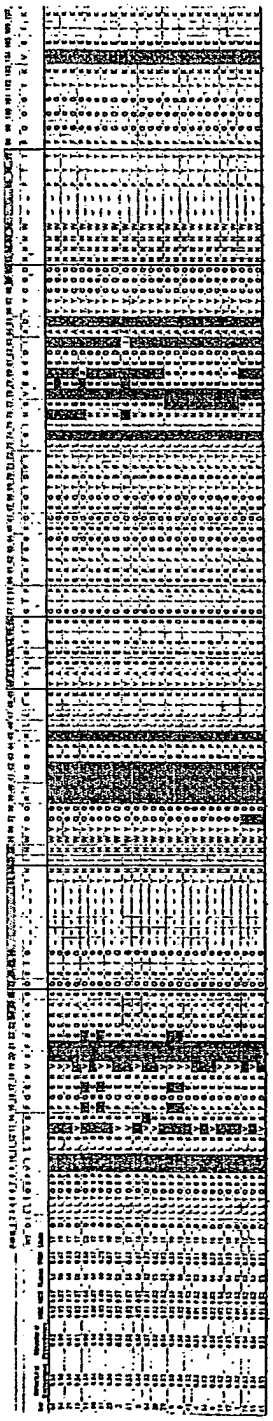

Figure 30a. C225 VL HEC Calculation 1

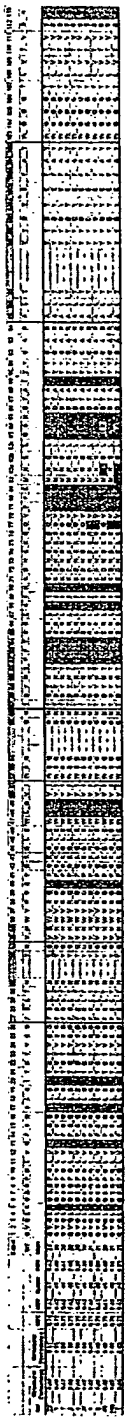

Figure 30b. C225 VH HEC Calculation 1

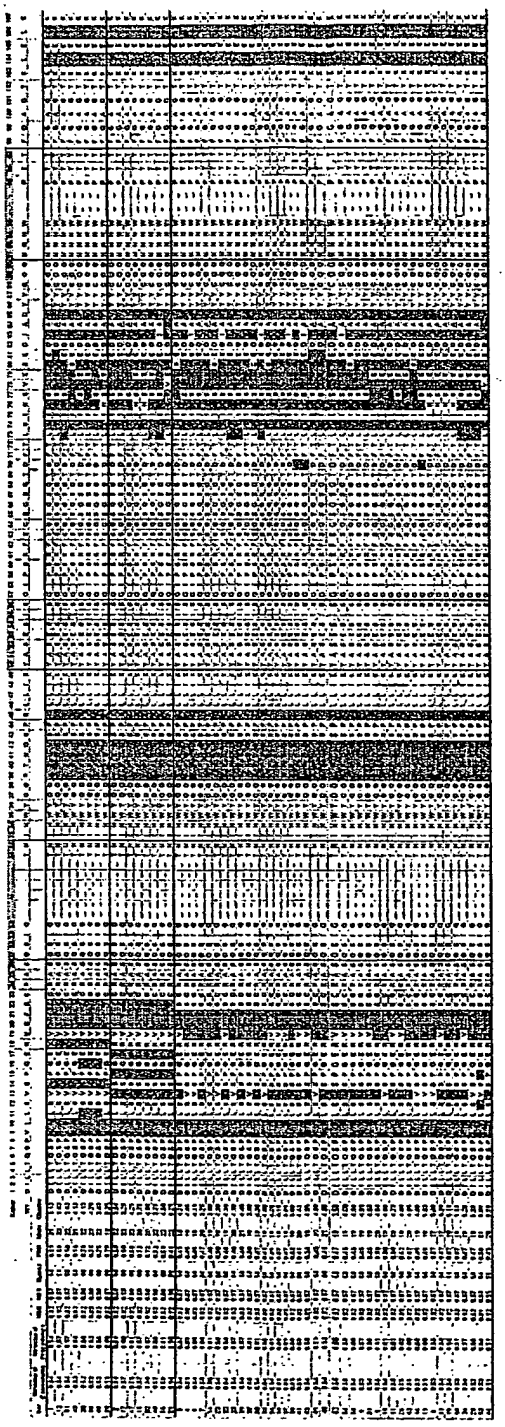

Figure 31a. C225 VL HEC Calculation 2

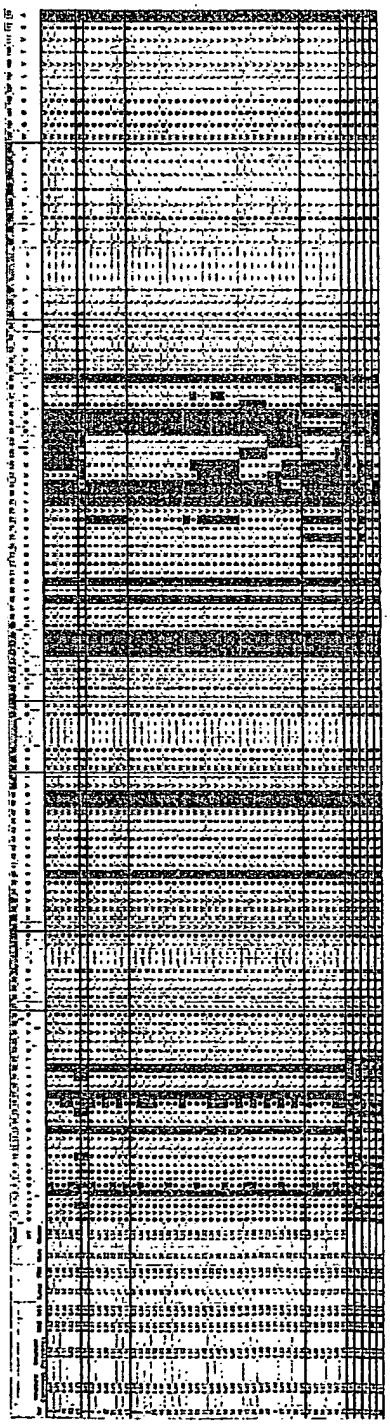

Figure 31b. C225 VH HEC Calculation 2

Figure 32. L2 C225 VL

Figure 32a

DILLTQSP**AT**LS**L**SPGERV**TL**SCRASQSIGTNIHWYQQ**KPDQ**SP**K**LLIKYASESISGIPSRFS
GSGSGTDFTL**TISSLQ**PEDFADYYCQQNNNWPTTFGAGTKLE**I**K

Figure 32b

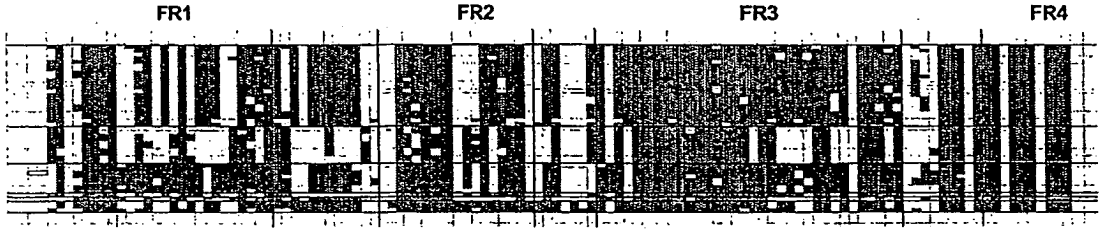

Figure 32c

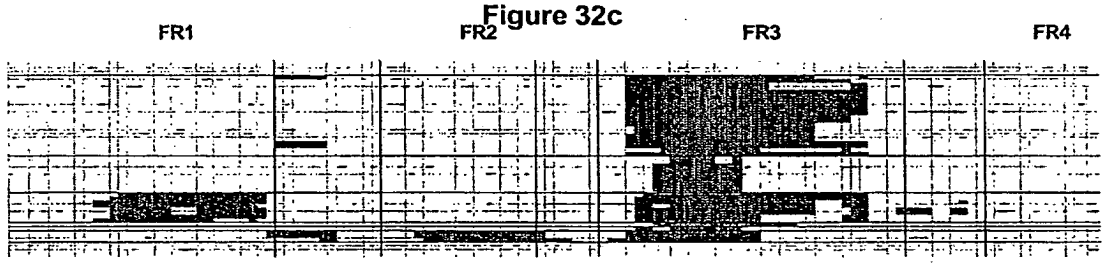

Figure 32d

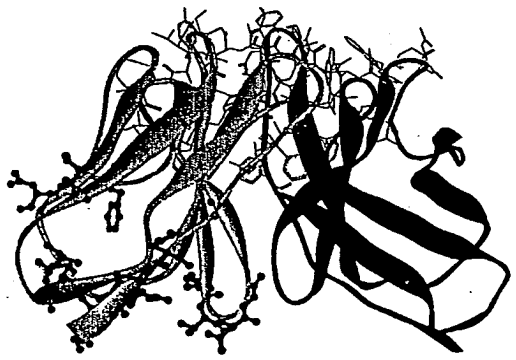

Figure 33. L3 C225 VL

Figure 33a

DILLTQSP**SS**LS**A**SVG**D**RV**TIT**CRASQSIGTNIHWYQQ**KPDQ**SP**K**LLIKYASESISGIPSRFS
GSGSGTDFTL**TISSLQA**EDVA**V**YYCQQNNNWPTTFGAGTKLEIK

Figure 33b

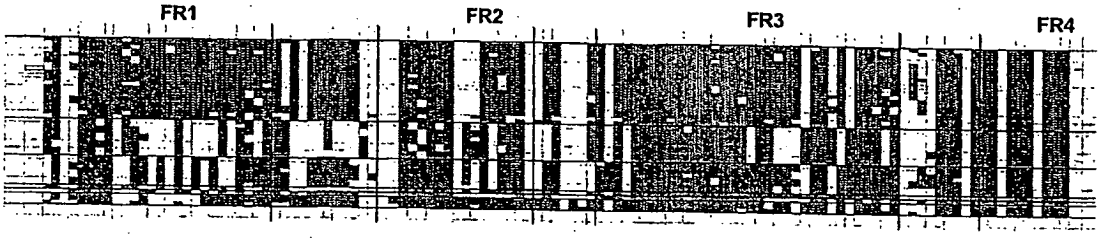

Figure 33c

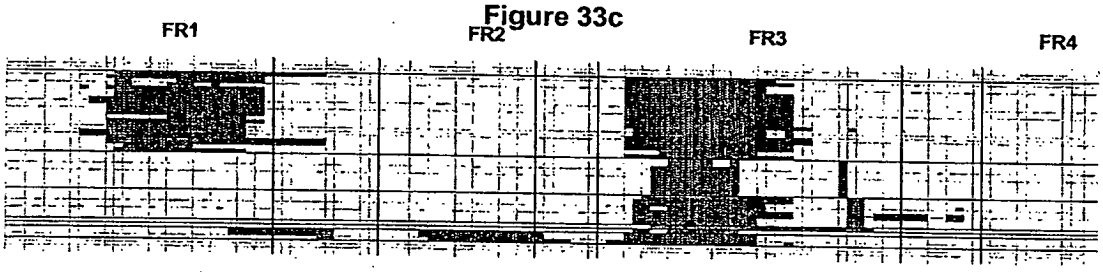

Figure 33d

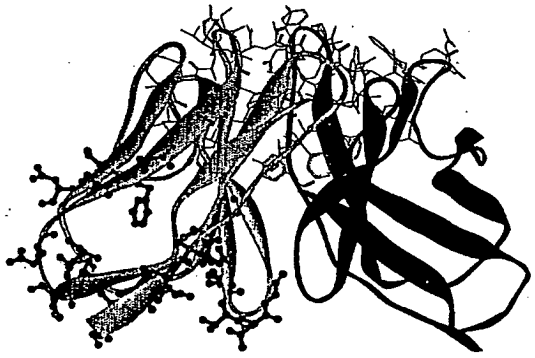

Figure 34. L4 C225 VL

Figure 34a

DILLTQSPAFLSVTPGEKVTITCRASQSIGTNIHWYQQKPDQSPKLLIKYASESISGIPSRFSG
SGSGTDFTLTINSLEAEDAATYYCQQNNNWPTTFGAGTKLEIK

Figure 34b

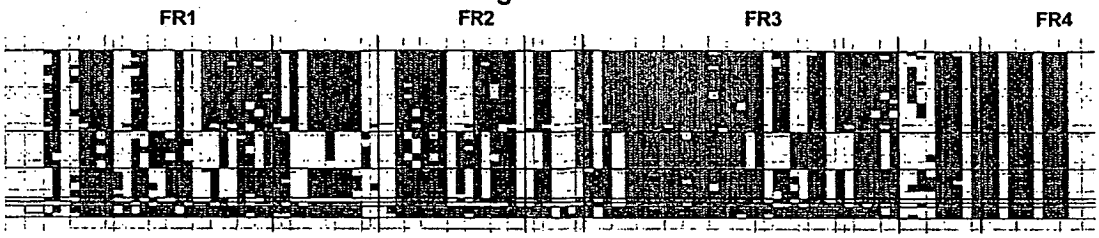

Figure 34c

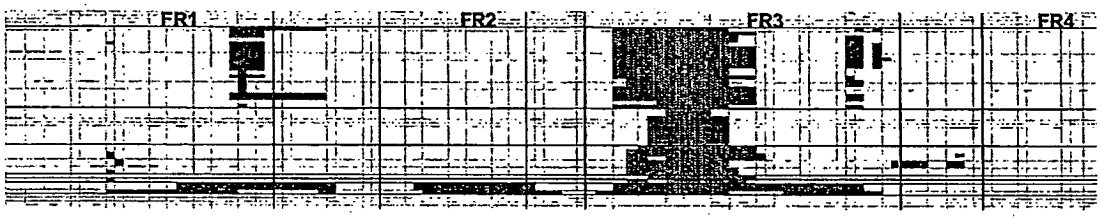

Figure 34d

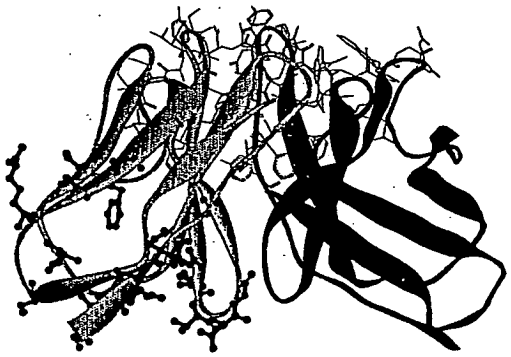

Figure 35. H3 C225 VH

Figure 35a

QVQLQQSGPGLVKPSQTLSLTCTVSGFSLTNYGVHWVRQAPGKGLEWLGVIWSGGNTDY
NTSLKSRLTISKDNSKSQVVLQMNSLRAEDTAVYYCARALTYYDYEFAYWGQGTLVTVSS

Figure 35b

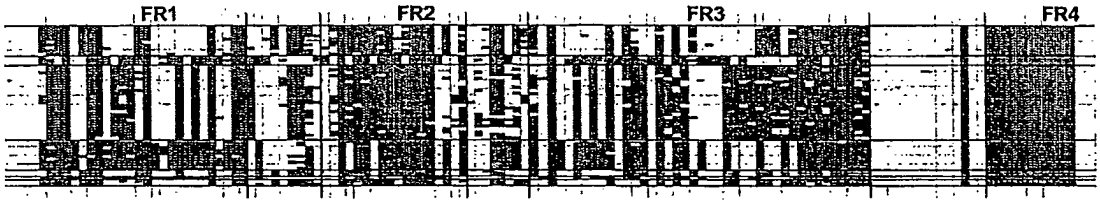

Figure 35c

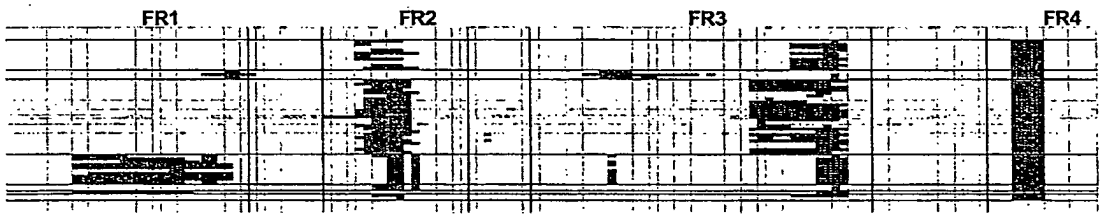

Figure 35d

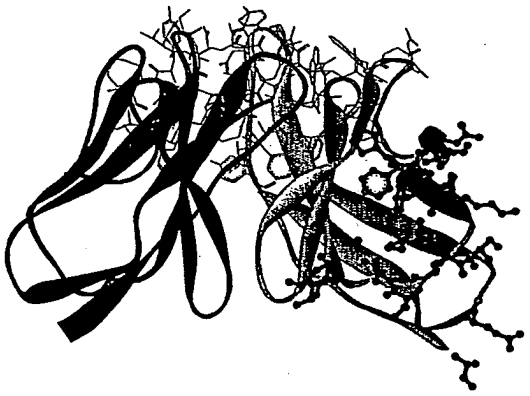

Figure 36. H4 C225 VH

Figure 36a

QVQL**Q**QSGPGLV**K**PSQ**T**LS**L**TCTVSGFSLTNYGVHWVRQ**A**PGKGLEWLGVIWSGGNTDY
NT**SLK**SRL**T**I**S**KDNSKSQV**VLT**M**TNMDPV**DTA**T**YYCARALT**Y**YDYEFAYWGQGTLVTV**SS**

Figure 36b

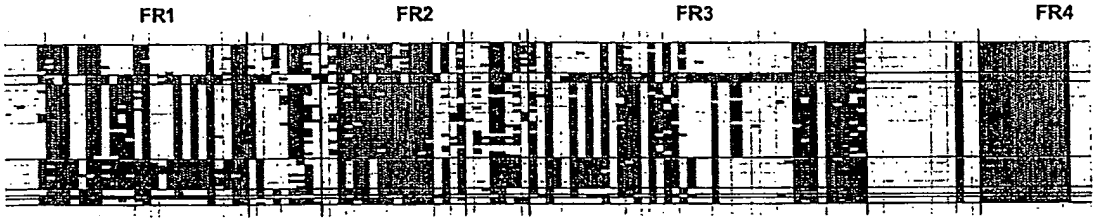

Figure 36c

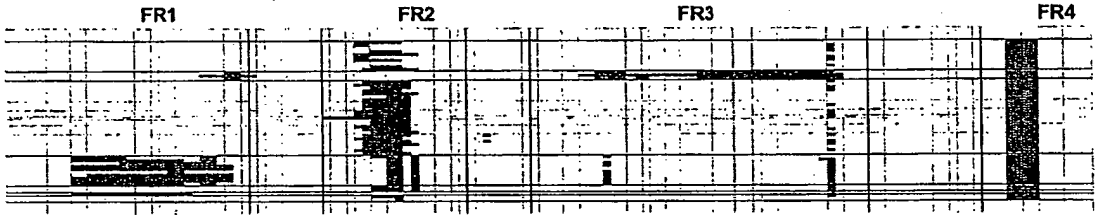

Figure 36d

Figure 37. H5 C225 VH

Figure 37a

QVQLQQSGPGLVKPSQTLSLTCTVSGFSLTNYGVHWVRQAPGKGLEWLGVIWSGGNTDY
NTPLTSRLTINKDNSKSQVVLQMNSLRAEDTAVYYCARALTYYDYEFAYWGQGTLVTVSS

Figure 37b

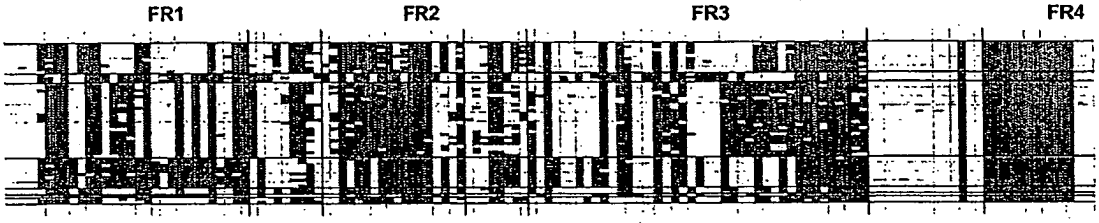

Figure 37c

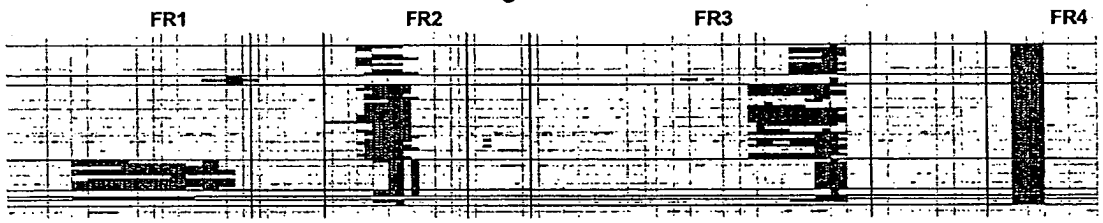

Figure 37d

Figure 38. H6 C225 VH

Figure 38a

QVQLQQSGPGLVKPSQTLSLTCTVSGFSLTNYGVHWVRQAPGKGLEWVGVIWSGGNTDY
NTSVKGRFTISKDNSKSQVYLQMNSLRAEDTAVYYCARALTYYDYEFAYWGQGTLVTVSS

Figure 38b

Figure 38c

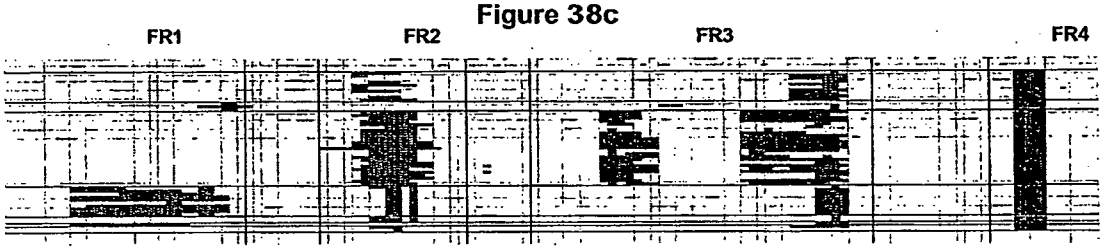

Figure 38d

Figure 39. H7 C225 VH

Figure 39a

QVQLQQSGPGLVKPSQTLSLTCTVSGFSLTNYGVHWVRQPPGKGLEWIGVIWSGGNTDY
NTSLKSRVTISKDNSKSQVSLKLSSVTAADTAVYYCARALTYYDYEFAYWGQGTLVTVSS

Figure 39b

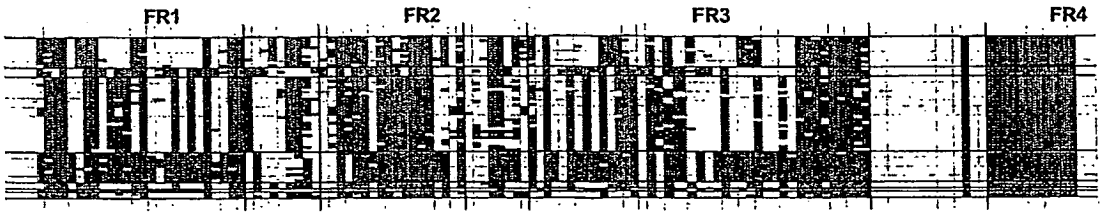

Figure 39c

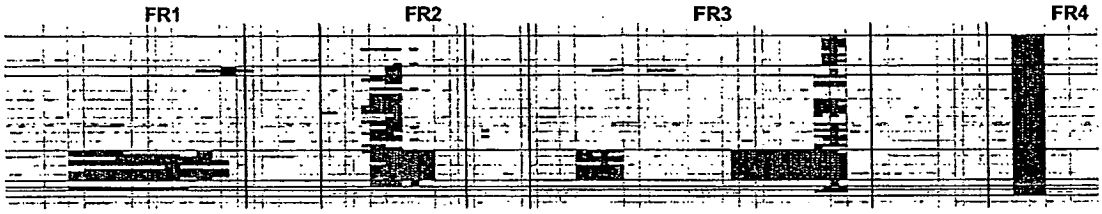

Figure 39d

Figure 40. H8 C225 VH

Figure 40a

QVQL**VE**SG**G**GLVQP**GR**SL**RL**S**CA**VSGFSLTNYGVHWVRQ**A**PGKGLEW**VS**VIWSGGNTD
YNT**SVKG**R**FTIS**KDNSKS**TVYLQ**MNSL**RAE**DTA**VYYC**A**RALTYYDYEFAYWGQGTLVTVS
S

Figure 40b

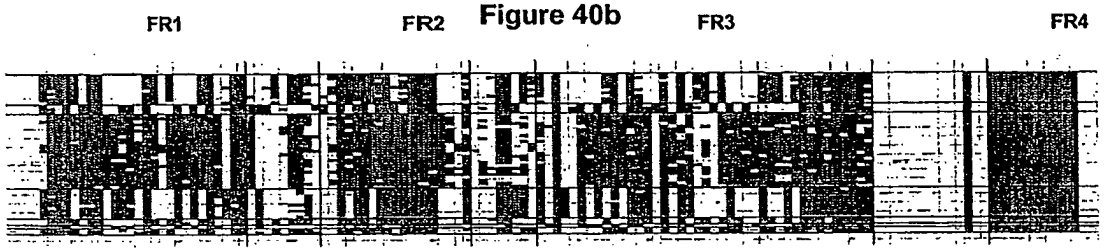

Figure 40c

Figure 40d

Figure 41

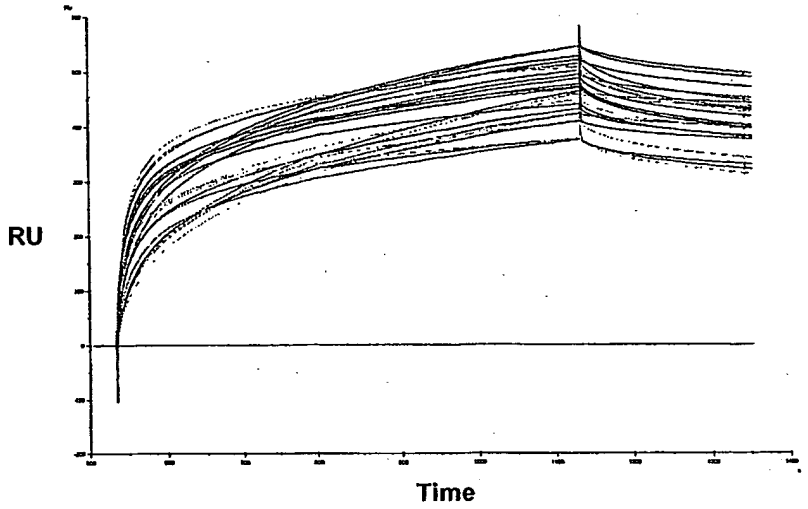

Figure 42a

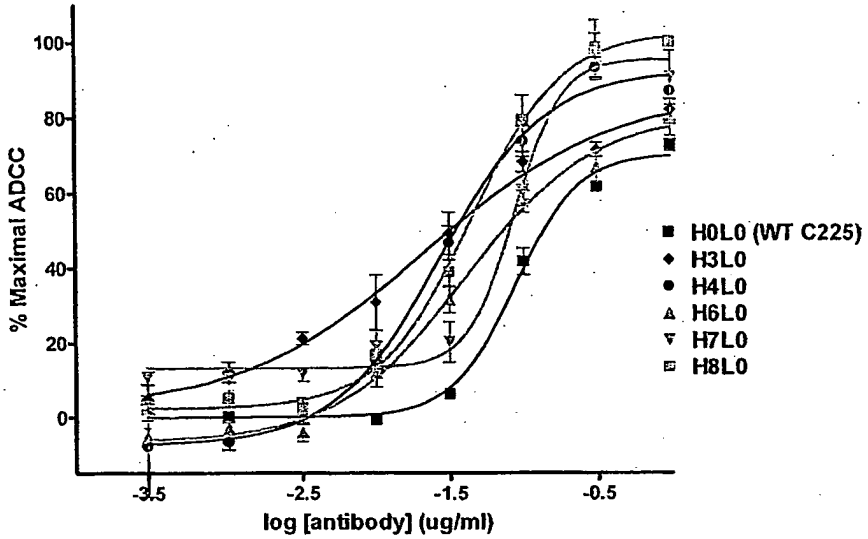

Figure 42b

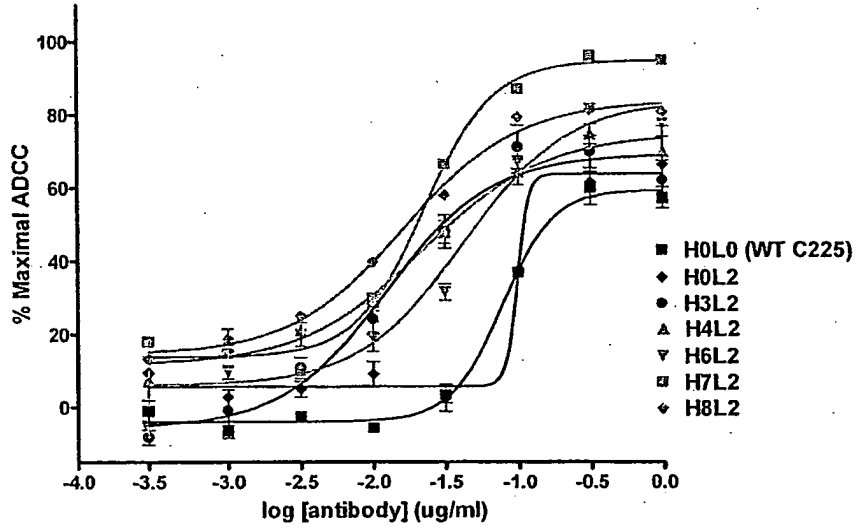

Figure 42c

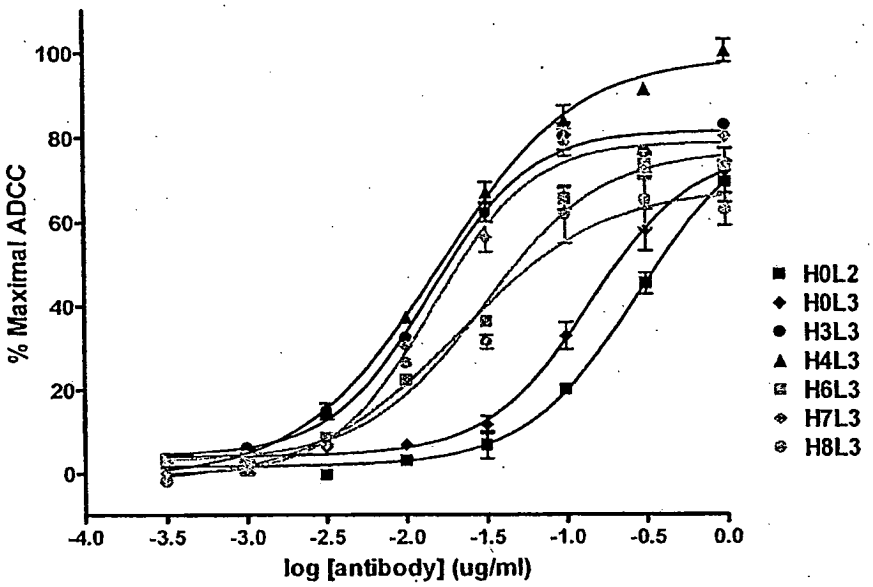

Figure 42d

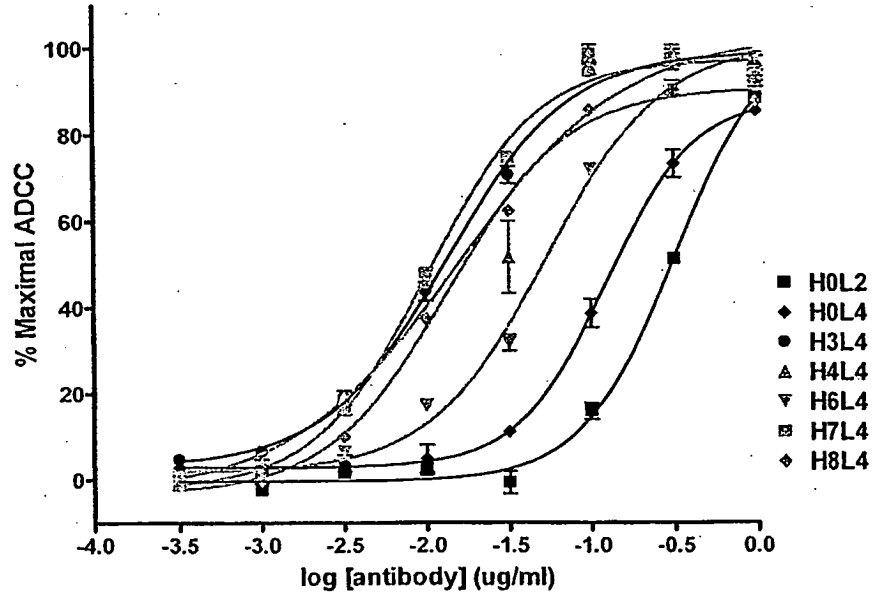

Figure 43. WT ICR62 VL

Figure 43a

DIQMTQSPSFLSASVGDRVTINCKASQNINNYLNWYQQKLGEAPKRLIYNTNNLQTGIPSRF
SGSGSGTDYTLTISSLQPEDFATYFCLQHNSFPTFGAGTKLELK

Figure 43b

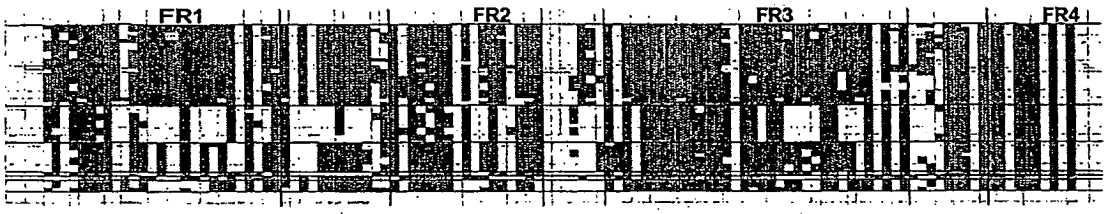

Figure 43c

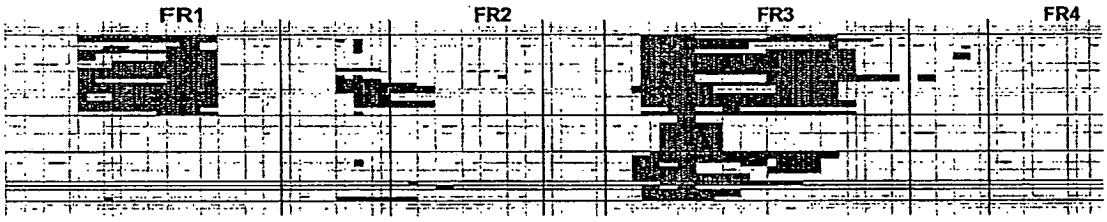

Figure 43d

Figure 44. WT ICR62 VH

Figure 44a

QVNLLQSGAALVKPGASVKLSCKGSGFTFTDYKIHWVKQSHGKSLEWIGYFNPNSGYSTY
NEKFKSKATLTADKSTDTAYMELTSLTSEDSATYYCTRLSPGGYYVMDAWGQGASVTVSS

Figure 44b

Figure 44c

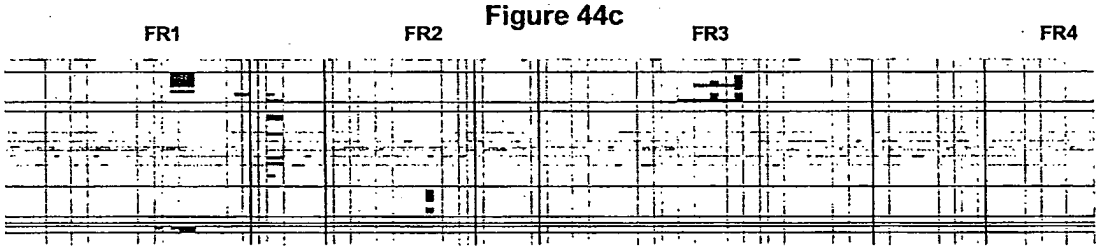

Figure 44d

Figure 45. CDR grafted ICR62 VL

Figure 45a

DIQMTQSPSSLSASVGDRVTITCRASQNINNYLGWYQQKPGKAPKRLIYNTNNLQTGVPSR
FSGSGSGTEFTLTISSLQPEDFATYYCLQHNSFPTFGAGTKLEIK

Figure 45b

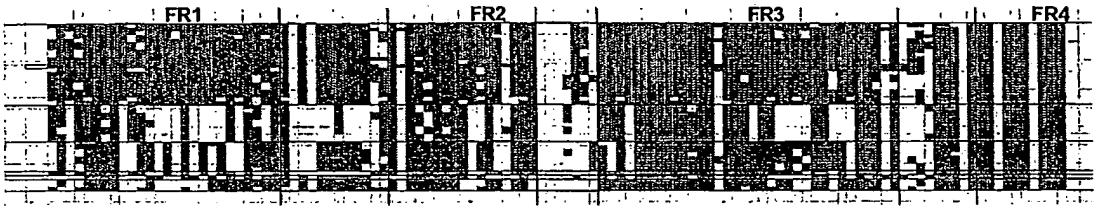

Figure 45c

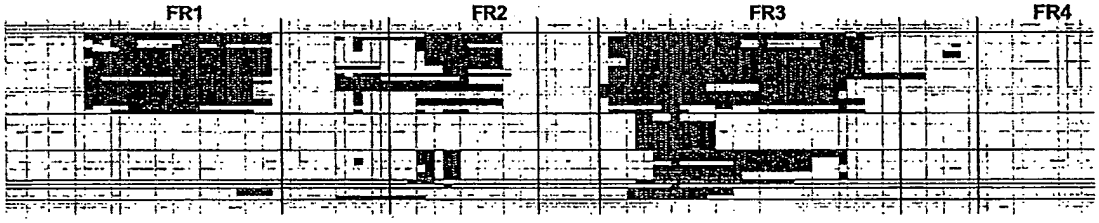

Figure 45d

Figure 46. CDR grafted ICR62 VH

Figure 46a

EVQLVQSGAEVKKPGATVKISCKVSGFTFTDYKMHWVQQAPGKGLEWMGLVNPNSGYTI
YAEKFQGRVTITADTSTDTAYMELSSLRSEDTAVYYCATLSPGGYYVMDAWGQGTLVTVS
S

Figure 46b

Figure 46c

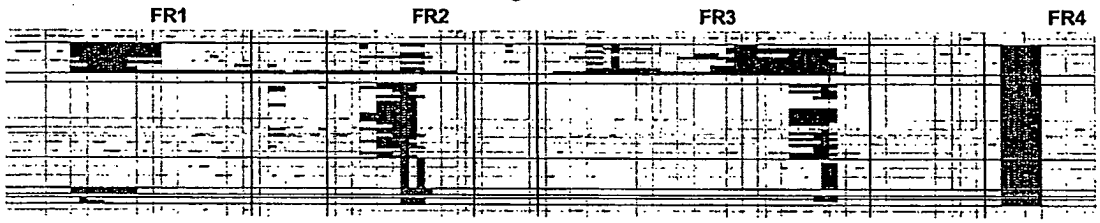

Figure 46d

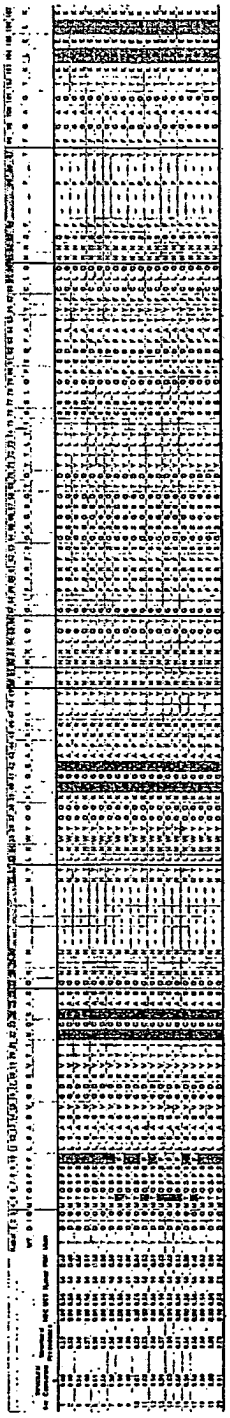

Figure 47a. ICR62 VL HEC Calculation 1

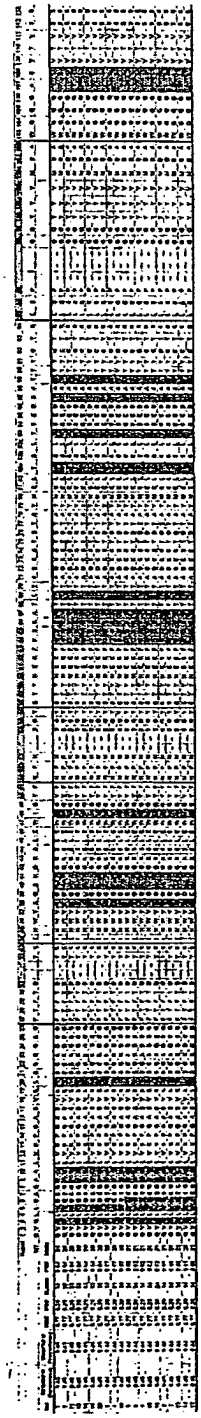

Figure 47b. ICR62 VH HEC Calculation 1

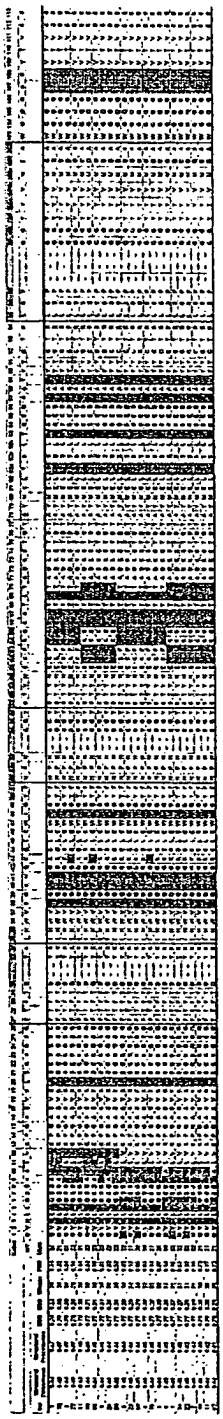

Figure 47c. ICR62 VH HEC Calculation 2

Figure 48. L3 ICR62 VL

Figure 48a

DIQMTQSPSSLSASVGDRVTITCRASQNINNYLNWYQQKPGKAPKRLIYNTNNLQTGIPSRF
SGSGSGTDYTLTISSLQPEDFATYFCLQHNSFPTFGAGTKLEIK

Figure 48b

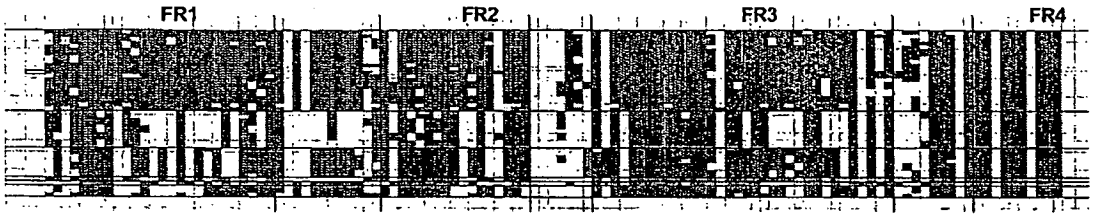

Figure 48c

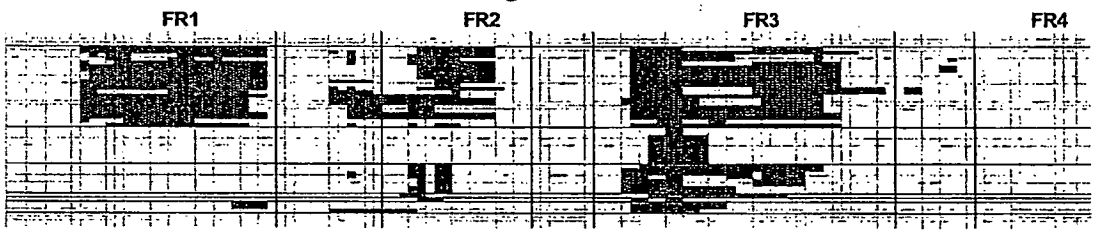

Figure 48d

Figure 49. H9 ICR62 VH

Figure 49a

QV**Q**L**Q**QSG**PG**LVKPGASVK**V**SCKGSGFTFTDYKIHWV**RQAP**GKSLEW**M**GYFNPNSGYST
YNEKF**QGRV**TI**T**ADKSTDTAYMEL**S**SL**R**SEDT**AV**YYCTRLSPGGYYVMDAWGQG**TL**VTVS
S

Figure 49b

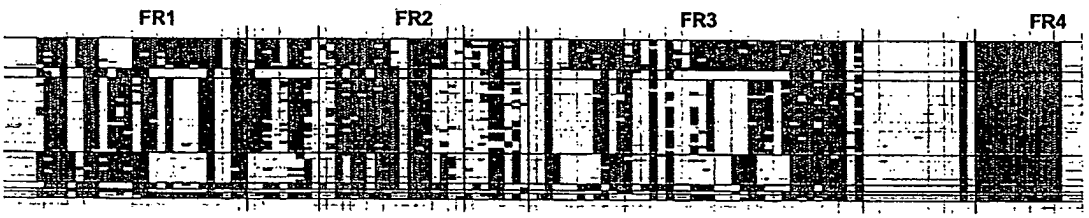

Figure 49c

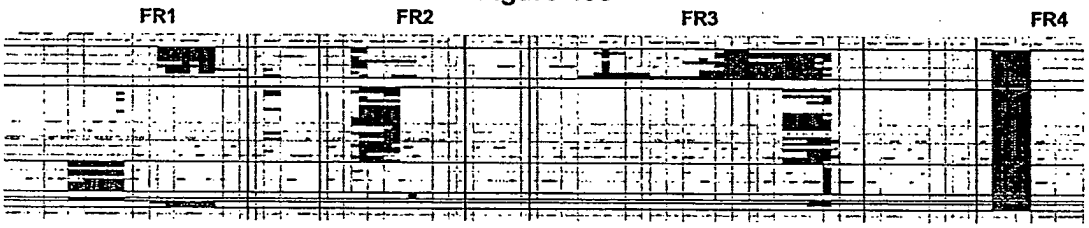

Figure 49d

Figure 50. H10 ICR62 VH

Figure 50a

QV**Q**LVQSGA**EVK**KPGASV**K**V**S**CKGSGFTFTDYKIHWV**R**Q**AP**GKSLEW**M**GYFNPNSGYST
YNEKF**QGRVTI**TADKSTDTAYMEL**S**SL**R**SEDTAVYYCTRLSPGGYYVMDAWGQG**TL**VTVS
S

Figure 50b

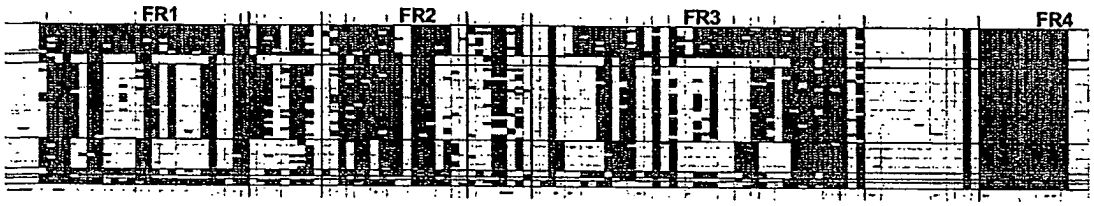

Figure 50c

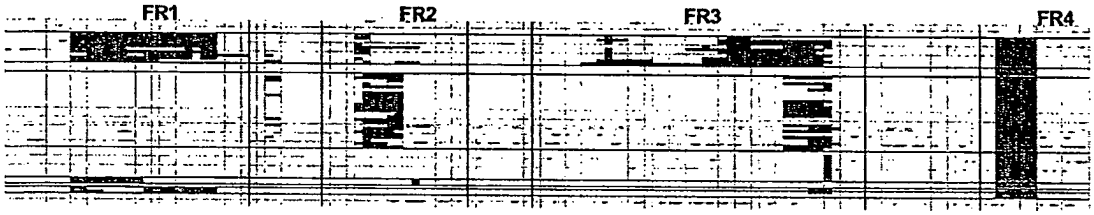

Figure 50d

15.14 Confidentiality.

(a) No Limited Partner shall disclose to any Person any information related to the General Partner, the Partnership, any Principal, any Parallel Investment Vehicle, any Alternative Investment Vehicle, any Portfolio Company or proposed Portfolio Company or any of their respective Affiliates, in each case, that is not publicly available (or that is publicly available as a result of a disclosure by such Limited Partner or any director, employee, officer, agent, legal, financial or tax advisor of such Limited Partner in violation of this Section 0~~15.14~~); provided, however, that nothing contained herein shall prevent any Limited Partner from furnishing (i) any ~~required~~ information that such Limited Partner is required to provide to any governmental regulatory agency, self-regulating body or in connection with any judicial, governmental or other regulatory proceeding or as otherwise required by any applicable law, rule or regulation (provided that any disclosure that is either (x) not to a governmental regulatory agency or (y) not on a confidential basis, shall require prior written notice thereof to the General Partner) or (ii) any information, so long as such disclosure is for a bona fide business purpose relating to the such Limited Partner's investment in the Partnership ~~of such Limited Partner~~, to directors, officers, employees and legal, financial and tax advisors of such Limited Partner who are informed of the confidential nature of the information and who agree to be bound by the provisions of this Section 0~~15.14~~ or who are otherwise bound by substantially similar obligations of confidentiality, and each Limited Partner agrees to ~~be bound hereby~~ impose such requirements and shall be liable for any breach of this Section 15.14 by such persons. Without limitation of the foregoing, each Limited Partner acknowledges that notices and reports to such Limited Partner hereunder may contain material non-public information concerning, among other things, Portfolio Companies, and agrees not to use such information other than in connection with monitoring its investment in the Partnership and agrees, in that regard, not to trade in publicly traded Securities on the basis of any such information. Furthermore, the Partners hereby acknowledge that pursuant to § 17-305(f) of the Act, the rights of a Limited Partner to obtain information from the Partnership shall be limited to only those rights provided for in this Agreement, and that any other rights provided under § 17-305(a) of the Act shall not be available to the Limited Partners or applicable to the Partnership.

Comment [M1]: needs work.

(b) In order to preserve the confidentiality of certain information disseminated by the General Partner or the Partnership under this Agreement that a FOIA Limited Partner ~~that is subject to FOIA or any Limited Partner that has one or more equity owners that are subject to FOIA (any such Limited Partner, a "*FOIA Limited Partner*")~~ is entitled to receive pursuant to the provisions of this Agreement, including, without limitation, quarterly, annual and other reports (other than Schedule K-1s), information provided to the Advisory Committee and any information provided at meetings of the Limited Partners, the General Partner may (i) provide to such FOIA Limited Partner access to such information only on the Partnership's (or Management Company's) website in password protected, non-downloadable, non-printable format, ~~or~~ (ii) require such FOIA Limited Partner to return any copies of information provided to it by the General Partner or the Partnership (including any subsequent copies made by such Limited Partner) to the extent not prohibited by applicable law or regulation or (iii) [redacted].

Comment [M2]: Move to definitions

Comment [M3]: - add mutual agreement on satisfactory method including visits

(c) Notwithstanding the provisions of Section 0~~15.14~~, the General Partner agrees that each Limited Partner that (x) is a private fund of funds (or other similar private collective investment vehicle) having reporting obligations to its investors and (y) has, prior to the date on

which such Limited Partner was admitted to the Partnership, notified the General Partner in writing that it is electing the benefits of this Section 1.1(c)~~15.14(c)~~ may, in order to satisfy such reporting obligations, provide the following information to its investors (but only to the extent that such investors are informed of the confidential nature of the information and either agree to be bound by the provisions of this Section 0~~15.14~~ or are otherwise bound by substantially similar obligations of confidentiality): (i) the name and address of the Partnership; (ii) the fact that such Limited Partner is a limited partner of the Partnership and the Partnership's general investment strategy; (iii) the identity of the General Partner and any Principal; (iv) the final closing date of the Partnership; (v) the amount of such Limited Partner's Commitment; (vi) the total amount of such Limited Partner's Capital Contributions; (vii) the total amount of distributions received by such Limited Partner from the Partnership; (viii) such Limited Partner's net internal rate of return with respect to the Partnership's performance as a whole as prepared by such Limited Partner; [(ix) the name of any Portfolio Company, a description of the business of such Portfolio Company and information regarding the industry and geographic location of such Portfolio Company; and (x) the cost of the Partnership's investment in a Portfolio Company]. With respect to any disclosure referred to in clauses (i) through (x) above, each Limited Partner shall indicate that such disclosure was not prepared, reviewed or approved, by the General Partner or the Partnership.

Comment [M4]: Should not that this election be included in subscription agrement

Comment [M5]: Expand list: management fees, date of investment, public liquidity events

Comment [M6]: Add assurance that this paragraph does confer any right to receive such information

(d) Each Limited Partner shall promptly notify the General Partner if at any time such Limited Partner is or becomes subject to Section 552(a) of Title 5 of the United States Code (commonly known as the "Freedom of Information Act") or any public disclosure law, rule or regulation of any governmental body or non-governmental regulatory entity that could require similar or broader public disclosure of confidential information provided to such Limited Partner (collectively such laws, rules or regulations, "*FOIA*"). To the extent that any such Limited Partner receives a request for public disclosure of any confidential Partnership information provided to it, such Limited Partner agrees that: (i) it shall use its reasonable best efforts to (~~x~~w) promptly notify the General Partner of such disclosure request and promptly provide the General Partner with a copy of such disclosure request or a detailed summary of the information being requested, (~~y~~x) inform the General Partner of the timing for responding to such disclosure request, (~~z~~y) consult with the General Partner regarding the response to such disclosure request; (~~ii~~z) ~~it shall use commercially reasonable efforts to~~ oppose and prevent the requested disclosure unless (A) such Limited Partner ~~is advised~~determines in good faith upon advice of ~~by~~ counsel that there exists no reasonable basis on which to oppose such disclosure or (B) such disclosure relates solely to the information contained in clauses (i) through (~~x~~viii) of Section 1.1(c)~~15.14(c)~~ (and does not include any information relating to individual Portfolio Companies and/or copies of this Agreement or related documents); and (iii) notwithstanding any other provision of this Agreement, the General Partner may, in order to prevent any such potential disclosure that the General Partner determines in good faith is likely to occur, withhold all or any part of the information otherwise to be provided to such Limited Partner; provided, however, that the General Partner shall not withhold any such information if such Limited Partner confirms in writing to the General Partner, based upon advice of counsel, that compliance with the procedures in Section 1.1(b)~~15.14(b)~~ is legally sufficient to prevent such potential disclosure.

Comment [M7]: Update if additional items added but exclude any portfolio company information if listed above

Comment [M8]: Any lower standard?

(e) The obligations and undertakings of each Limited Partner under this Section 0~~15.14~~ shall be continuing and shall survive termination of the Partnership and this Agreement. Any restriction or obligation imposed on a Limited Partner pursuant to this Section 0~~15.14~~ may

be waived by the General Partner in its discretion. Any such waiver or modification by the General Partner shall not constitute a breach of this Agreement or of any duty stated or implied in law or in equity to any Limited Partner, regardless of whether different agreements are reached with different Limited Partners.

(f) The parties hereto agree that irreparable damage would occur if the provisions of this Section 015.14 were breached. It is accordingly agreed that the parties hereto shall be entitled to ~~seek~~ an injunction or injunctions to prevent breaches of this Section 015.14 and to enforce specifically the terms and provisions hereof in any court of the U.S. or any state having jurisdiction, in addition to any other remedy to which they are entitled at law or in equity.

Comment [M9]: add concept that also agree not to raise as a defense

Formatted: Bullets and Numbering

(g) Add Tax Carve out

(h) Add Delaware 305(b) boilerplate about ability to withhold

EXHIBIT K

Pipeline and Utility Easements Deed – by Owners to Mineral Owners and Mineral Lessee

RECORDING REQUESTED BY: Chicago Title Company WHEN RECORDED MAIL TO: Esperson/Grimm c/o Kronick Moskovitz Tiedemann & Girard 1675 Chester Avenue, Suite 320 Bakersfield, CA 93301 Attention: Teri A. Bjorn, Of Counsel.	

The undersigned Grantors declare:

Documentary transfer tax is: $_____.

( ) computed on full value of property conveyed, or

( ) computed on full value less value of liens and encumbrances remaining at time of sale.

( ) Unincorporated area of County of Solano, or

( ) City of Rio Vista.

GRANT OF PIPELINE AND UTILITY EASEMENTS

THIS GRANT OF PIPELINE AND UTILITY EASEMENTS (this "Easement Deed") is made and entered into as of _____, 200__ (the "Effective Date") by and among:

(1) **THE GRIMM-RIO VISTA FAMILY LIMITED PARTNERSHIP,** a California limited partnership; **RICHARD W. ESPERSON,** also known as **RICHARD W. ESPERSON, JR.,** and **IRENE S. ESPERSON, TRUSTEES OF THE RICHARD W. ESPERSON AND IRENE SUE ESPERSON FAMILY TRUST DATED OCTOBER 17, 1991; MARK ESPERSON; GARY ESPERSON; STEPHEN ESPERSON;** and **KIMBERLY ESPERSON** (collectively the "Surface Owners"), the fee owners of that certain real property legally described in Exhibit A-1 (the "Property").

(2) **RIO VISTA HILLS HOLDING COMPANY, LLC,** a Delaware limited liability company ("RVHHC"), who has an option to purchase that portion of the Property shown and identified on Exhibit B as the Sale Property (the "Sale Property"). The remainder of the Property shown and identified on Exhibit B, other than the Sale Property, is referred to as the "Retained Property." Surface Owners and RVHHC are collectively sometimes referred to herein as the "Grantors."

(3) **NORMA JEAN GRIMM,** also known as **JEAN HARRIS GRIMM, AS TRUSTEE OF THE TRIMM FAMILY TRUST DATED OCTOBER 4, 1990 – SURVIVORS TRUST; RICHARD W. ESPERSON, JR.; JOAN ESPERSON WEDDELL; DAVID SANTOS; RICHARD SANTOS; STEPHEN ESPERSON; GARY ESPERSON; MARK ESPERSON; SUSAN A. BORGESEN,** formerly **SUSAN A. WOODWORTH; SANDRA J. DICKSON,** formerly **SANDRA GRIMM; SHARON E. HARRIS,** formerly **SHARON GRIMM; STEPHEN A. GRIMM,** formerly **STEPHEN GRIMM; THE GRIMM-RIO VISTA FAMILY LIMITED PARTNERSHIP,** a California limited partnership; **JENA HARRIS GRIMM; DAVID L. SANTOS, SURVIVING TRUSTEE OF THE DAVID L. AND LAURA E. SANTOS REVOCABLE TRUST DATED FEBRUARY 12, 2002;** and **RICHARD W. ESPERSON,** also known as **RICHARD W. ESPERSON, JR.,** and **IRENE S. ESPERSON, TRUSTEES OF THE RICHARD W. ESPERSON AND IRENE SUE ESPERSON FAMILY TRUST DATED OCTOBER 17, 1991** (collectively the "Mineral Owners," each being a "Mineral Owner"), the owners of the reversionary interest in that certain mineral estate (the "Mineral Estate") in, on and under the Property and comprised of that portion of Parcel One and all of Parcels Two, Three and Four shown within bold line on the diagram attached as Exhibit C-1, which owners are listed as to each of such Parcels One, Two, Three and Four in Exhibit C-2. The Mineral Estate is legally described in Exhibit C-3.

(4) The owner of the fee simple determinable interest in the Mineral Estate (by virtue of the grant of oil and gas lease concerning the Mineral Estate), **ROSETTA RESOURCES OPERATING, L.P.,** a Delaware limited partnership, successor to Calpine Natural Gas California, Inc., and Sheridan California Energy, Inc., both California corporations (the "Mineral Lessee"). For this Easement Deed, the exclusive fee simple determinable interest held by Mineral Lessee may be referred to as a mineral leasehold interest. The mineral leasehold estate of Mineral Lessee also includes rights in and to the real property located outside of the bold line on Exhibit B-1 (the "Adjacent Property") and rights in and to that mineral estate in, on and under the Adjacent Property (the "Adjacent Mineral Estate"), which together with Mineral Lessee's leasehold rights in and to the Property and the Mineral Estate is referred to collectively as the "Leasehold Estate." The Leasehold Estate encumbers all of the Parcels shown on Exhibit C-1 and is legally described in Exhibit C-4. Mineral Owners and Mineral Lessee are collectively referred to herein sometimes as the "Grantees."

Surface Owners, RVHHC, Mineral Owners and Mineral Lessee are sometimes collectively referred to herein as the "Parties" or individually as a "Party."

RECITALS:

A. Surface Owners own the Property, excepting the Mineral Estate, which is comprised of approximately 504 assessed acres of undeveloped real property in the City of Rio Vista ("City"), County of Solano, State of California, Assessor's Parcel Nos. 49-310-040, 49-310-300, 49-310-020 and 49-310-010. A diagram of the Property is attached as Exhibit B.

B. Surface Owners have entered into an Option Agreement (the "Option Agreement") with RVHHC to sell the Sale Property for development of a residential/commercial project, including school sites, recreation/park sites, and open space areas and public uses (the "Project") as generally shown on Exhibit D. The Retained Property shown on Exhibit B will be retained for development by Surface Owners as part of an overall master-planned community to be known as Del Rio Hills, which is currently planned to contain only the Sale Property and the Retained Property. This Easement Deed covers the Property which includes both the Sale Property and the Retained Property.

C. As used in this Easement Deed, "Owner" means both Surface Owner and RVHHC and their respective heirs, assigns, transferees and successors. However, for purposes of apportioning responsibility and liability for the obligations generally ascribed to "Owner" in this Easement Deed, and unless the context of this Easement Deed otherwise specifically provides, (i) the fee estate owner of the Sale Property shall be responsible and liable only for those obligations, actions and activities arising under this Easement Deed that pertain to or otherwise stem from the ownership of the Sale Property and (ii) the fee estate owner of the Retained Property shall be responsible and liable only for those obligations, actions and activities arising under this Easement Deed that pertain to or otherwise stem from the ownership of the Retained Property. If the Option Agreement terminates or is terminated prior to RVHHC acquiring any portion of the Property, the term RVHHC shall mean Surface Owners or future developers taking their interest through Surface Owners, and RVHHC shall not have any further rights under the Accommodation Agreement (as defined in Recital G herein) or this Easement Deed.

D. All oil, natural gas, casinghead gas, condensate and other hydrocarbon substances in, on and under the Property are part of the Mineral Estate.

E. Mineral Lessee is the successor lessee under an Oil and Gas Lease between Edward Drouin, also known as E. D. Drouin, as lessor, and Amerada Petroleum Corporation of California, as lessee, dated May 28, 1935 (the "Lease"), and recorded on October 9, 1935, in Book 151, Page 72, Official Records of Solano County, California. The Lease covers 912 acres, including the entire Property (504 acres) and the Adjacent Property (408 acres). The Leasehold Estate granted to Mineral Lessee by the Lease includes a fee simple determinable interest in the Mineral Estate, including the exclusive right to prospect, explore, test (including seismic and geologic investigations), drill for, produce, mine, extract, transport and remove oil, natural gas, casinghead gas, and other hydrocarbon substances from the Property and the Adjacent Property. Pursuant to the Lease, Mineral Lessee has the right of ingress and egress to and from the surface of the Property and the Adjacent Property as necessary (consistent with the terms and limitations of the Lease and applicable law) to conduct prospecting, exploration, testing (including seismic and geologic investigations), drilling for, producing, mining, extracting, transporting and removing oil, natural gas, casinghead gas, and other hydrocarbon substances in, on or under the

Property and Adjacent Property. Said rights and activities are referred to herein as the "Extraction Operations."

F. The Mineral Owners are the owners of the reversionary interest to the Mineral Lessee's interest in the Mineral Estate and are the successors to the lessor of the Lease.

G. The Mineral Estate and the Leasehold Estate are subject to that certain Unit Agreement, Rio Vista Gas Unit, Contra Costa, Sacramento and Solano Counties, California, dated June 3, 1964, and recorded July 9, 1964, at Book 1280, Page 451, Official Records of Solano County (the "Unit Agreement"). Mineral Owners are parties to the Unit Agreement and receive a share of royalties for gas produced within the lands covered by the Unit Agreement, said lands defined as the "Unit Area" in the Unit Agreement. Mineral Lessee is the current Unit Operator, as defined in the Unit Agreement, and the current sole Working Interest Owner, as defined in the Unit Agreement. Mineral Lessee operates numerous existing natural gas wells in and below the Unit Area, and has the right under the Unit Agreement to conduct Extraction Operations within the Unit Area. Mineral Owners and Mineral Lessee's predecessors-in-interest entered into the Unit Agreement. Mineral Lessee has rights under the Unit Agreement, and various other leases, to explore, develop and extract natural gas, condensate and associated hydrocarbons within the Unitized Formation of the Unit Area as defined in the Unit Agreement, as well as the right of ingress and egress to and from the surface of the Unit Area as provided in the Unit Agreement and relevant leasehold agreements.

H. As of the Effective Date, Mineral Lessee has fourteen (14) existing gas well sites on the Property (each containing a well) and plans for six (6) additional well sites on the Property (these twenty (20) well sites are referred to collectively as the "Well Sites"). A Well Sites Plan shows the locations of all twenty (20) Well Sites, and is attached as Exhibit E. For purposes of this Easement Deed, each of the Well Sites is a "Well Site." The Well Sites will be used to support existing wells, and to drill new wells. Pipelines presently exist on the Property to support Mineral Lessee's existing Well Sites, and Mineral Lessee needs additional new pipelines on the Property to support the Well Sites (the pipelines presently existing on the Property and new pipelines planned by Mineral Lessee on the Property are referred to collectively as the "Pipelines"). Pursuant to the Lease and the Unit Agreement, Mineral Lessee also has numerous existing gas wells and pipelines located on the Adjacent Property, and has plans for additional wells and pipelines on the Adjacent Property.

I. Mineral Lessee is required to obtain certain approvals and/or permits ("City Extraction Approvals") from the City for the development of new natural gas facilities, including new wells and redrilling and deepening existing wells, pursuant to Chapter 13.12 of the City's Municipal Code ("Natural Gas Ordinance" or "NGO"). Mineral Lessee may also need to obtain approvals from the California Department of Conservation, Division of Oil, Gas and Geothermal Resources and possibly from other agencies such as the U.S. Fish and Wildlife Service and the California Department of Fish and Game for these drilling activities within the Well Sites ("Other Agency Extraction Approvals"). As used in this Easement Deed, the terms City Extraction Approvals and Other Agency Extraction Approvals shall concern only such drilling activities within the Property (and not drilling activities within the Adjacent Property for which Mineral Lessee is required to seek separate approvals).

J. In conjunction with the development of the surface of the Property for activities, structures and uses that are not related to mineral extraction ("Surface Development"), Owners are required to obtain approvals and/or permits from the City (including a subdivision map, pursuant to California Government Code Section 66410 *et seq.*), and possibly other governmental agencies, for Surface Development and related activities, construction and operations (collectively "Surface Development Approvals").

K. As a general matter, and subject to the terms and limitations of the Lease and applicable law, Mineral Lessee currently has non-exclusive rights to use the surface of the Property for the purposes set forth in the Lease and the Unit Agreement. Absent an agreement to specify which portion(s) of the Property can be used by which Party for what purpose(s), a possibility exists for conflict between and among the Parties as each exercises its rights in the Property. Accordingly, Mineral Owners and Mineral Lessee desired assurances and certainty that they will be able to continue existing Extraction Operations on certain portions of the Property, and in and below the Unit Area, under the terms and conditions of the Lease and the Unit Agreement and will be able to develop new Extraction Operations within the Well Sites, and in and below the Unit Area, under the terms and conditions of the Lease and the Unit Agreement. Similarly, Owners desire assurances and certainty that the Extraction Operations on the Property will be confined to certain areas of the Property, so that Owners can proceed with Surface Development.

L. The Parties' desired assurances and certainties are set forth in that certain Accommodation Agreement - Del Rio Hills ("Accommodation Agreement"), recorded on _____ at Series #_____ in the Official Records of Solano County.

M. Among other matters, the Accommodation Agreement (at Section 6.h) granted Mineral Owners and Mineral Lessee a temporary, unrestricted easement (the "Temporary Floating Pipeline/Utility Easement") over all of the Property for purposes of installing, operating and maintaining pipelines and related appurtenances necessary and convenient for Mineral Lessee's operations within the Property, the Unit Area and elsewhere. The Temporary Floating Pipeline/Utility Easement arose automatically and without the need for further documentation or approval upon the Parties' execution of the Accommodation Agreement; the Parties covenanted and agreed that the recordation of the Accommodation Agreement constituted sufficient and appropriate evidence of the grant of the Temporary Floating Pipeline/Utility Easement. The Temporary Floating Pipeline/Utility Easement will continue in full force and effect until such time as one or more final subdivision maps are recorded for Surface Development. Upon recordation of a final subdivision map for Surface Development, the Temporary Floating Pipeline/Utility Easement for only the portion of the Property which is the subject of the final subdivision map will automatically terminate and be of no further force or effect. The Temporary Floating Pipeline/Utility Easement for those portions of the Property which are not the subject of a final subdivision map for Surface Development will continue in full force and effect. Notwithstanding said automatic termination, Mineral Lessee and Mineral Owners will execute and deliver an appropriate instrument or document manifesting release of all or portions, as applicable, of the Temporary Floating Pipeline/Utility Easement following Owner's delivery

of (i) a written request for such instrument or document, and (ii) a copy of the recorded final subdivision map for Surface Development of the Property.

N. Pursuant to the Accommodation Agreement, Mineral Owners and Mineral Lessee desire to obtain exclusive easements across certain portions of the Property in order to establish, develop, maintain and repair and access Well Sites and to conduct Extraction Operations.

AGREEMENT:

In consideration of the mutual promises contained in this Easement Deed and of other valuable consideration, the receipt and sufficiency of which the Parties expressly acknowledge, it is agreed as follows:

1. Grant of Pipeline and Utility Easements.

Grantors hereby grant to Grantees temporary and permanent nonexclusive easements that are reasonably necessary for: (i) all pipelines and utilities necessary to service each Well Site, including without limitation, installation of conduits, pipe in pipe and wire in pipe; (ii) all Well Site flow lines and utility lines inside of protective conduit; (iii) pipeline stub-outs and utility or pipeline connections; and (iv) ingress and egress over and under the easement area for the installation, construction, operation, repair, maintenance, relocation and replacement of all pipelines and utilities (the "Pipeline/Utility Easements"). The Pipeline/Utility Easements shall be in gross and shall benefit Mineral Lessee and Mineral Owners, and are hereby conveyed to Mineral Owners and Mineral Lessee for non-exclusive use by Mineral Owners and Mineral Lessee. The Pipeline/Utility Easements as shown on the Well Sites Plan shows the Parties' agreed upon location of temporary and permanent easements which shall consist of (i) a permanent easement area a minimum of twenty (20) feet wide; and (ii) a temporary construction easement area to be used by Mineral Lessee during construction of pipelines and access roadways of an additional fifteen (15) feet on both sides of the twenty (20) foot permanent easement. The Pipeline/Utility Easements are designed to ensure, and shall have a scope sufficient to ensure, that any gas produced from any location (whether or not from below the Property, the Unit Area or elsewhere) that flows into the pipeline system on the Property has at least two outlets to exit the system on the Property.

2. Pipeline and Utility Construction.

a. If Mineral Lessee Constructs its Improvements in an Area Before Owners Construct their Improvements in the Same Area.

At all times, but prior to Surface Development in the vicinity of the area of the Property where Mineral Lessee desires to construct and install a pipeline or other utilities, Mineral Lessee shall be responsible for the installation and cost of all improvements necessary to transport production from its wells and to support its Well Sites with utility service. Mineral Lessee shall construct such improvements in the location of the Pipeline/Utility Easements set forth on the Well Sites Plan. Owners shall be entirely responsible to ensure that Mineral Lessee's improvements are not disturbed by any subsequent improvements, including grading, of the surface by Owners. In the event that Owners and Mineral Lessee agree, after Mineral Lessee

has installed pipelines and utilities, to modify the locations of any Pipeline/Utility Easements to accommodate Owners' Surface Development, then Owners shall be responsible for all costs of any resulting relocation of the pipelines and utilities. Mineral Lessee shall have the right to approve the time and manner of any relocation of pipelines or utilities done by Owners, or at Owners' request, prior to such relocation; such approval shall not be unreasonably withheld, conditioned or delayed, provided such relocation will not result in any substantial or material interruption of pipeline or utility service to Mineral Lessee's Extraction Operations on the Property. For purposes of this Easement Deed, "substantial or material interruption" shall mean a complete shut down, for a period exceeding forty-eight (48) consecutive hours, of one or more of Mineral Lessee's actively producing gas wells which utilized the affected Pipeline/Utility Easements, in spite of Mineral Lessee's commercially reasonable efforts to re-route gas production from the affected gas well(s) using other existing pipelines. Mineral Lessee shall issue its approval or disapproval within thirty (30) days of receipt of Owner's plans and specifications for the relocation of any pipelines or utilities. Mineral Lessee acknowledges and agrees that there will likely be temporary disruption of pipeline or utility service to the Extraction Operations on the Property when the new pipelines and utility connections are made. In the event that any such relocation activities result in a substantial or material interruption, Owner shall pay to Mineral Lessee a fee equal to Mineral Lessee's lost production value from the affected gas well(s) for the period (i) beginning at the expiration of the initial forty-eight (48) consecutive hours, and (ii) ending when the pipeline connection is fully restored to the actively producing gas well(s). For purposes of this Section 2.a, the fee payable to Mineral Lessee for the lost production value of an affected gas well shall be determined by the average of the affected gas well's daily yield for the thirty (30) day period preceding the date of the disruption, and based upon the contract price Mineral Lessee is then receiving from the purchaser of the gas well's production.

b. <u>If Owners Construct their Improvements in an Area before Mineral Lessee Constructs its Improvements in the Same Area</u>.

At all times after Owners have commenced development of the Property consistent with the Accommodation Agreement and the Surface Development Approvals on or near any of Mineral Lessee's Pipeline/Utility Easements, Owners shall install improvements (such as pipelines, water lines, telemetry/telephone lines and electrical lines) within the Pipeline/Utility Easements according to the Well Sites Plan or Survey, as appropriate (*see* Section 1.f of the Accommodation Agreement), to service existing or future wells at the Property as shown on the Well Sites Plan or Survey, as appropriate. Owners shall be responsible for the construction costs and installation of such pipelines and utilities; Mineral Lessee shall be responsible for the materials costs of the pipeline and other utility materials. The intent of this provision is to allow efficiencies of constructing Mineral Lessee's pipelines and other underground utilities at the same time Owners are building streets, sidewalks, and other infrastructure improvements. Prior to construction, Owners shall submit plans for such pipelines and other utility improvements to Mineral Lessee for Mineral Lessee's review and approval to ensure that the pipelines and improvements meet or comply with Mineral Lessee's specifications and standards and/or requirements of any approval or permit granted to Mineral Lessee for Extraction Operations. Mineral Lessee shall not unreasonably withhold, condition or delay approval of Owners' plans for such pipelines and other utility improvements, and Mineral Lessee shall issue its written approval or disapproval within thirty (30) days of receipt of Owners' plans

and specifications. If Mineral Lessee disapproves of the proposed plans, Mineral Lessee's written notice to Owners shall specify the nature of Mineral Lessee's objections and identify proposed revisions to the plans to correct the objections. Within thirty (30) days following receipt of Mineral Lessee's disapproval notice and proposed revisions, Owners shall notify Mineral Lessee in writing concerning whether Owners accept or reject the proposed revisions. If Owners accept the proposed revisions, the plans shall be duly modified and implementation of the pipeline improvements shall proceed accordingly. If Owners reject the proposed revisions, Owners and Mineral Lessee shall resolve their outstanding issues in accordance with the dispute resolution provisions set forth in Sections 12.a and 12.b of the Accommodation Agreement. [*This reference to the AA may work, as it relates to the construction phase, when AA still on title, prior to any conveyances of the areas burdened by the easements (open space, roads, etc.) to the City.*]

3. Pipeline and Utility Relocation.

a. Pipeline Location. Prior to the recordation of a final subdivision map for Surface Development, Mineral Lessee shall have the right to locate pipelines and utilities under the Temporary Floating Pipeline/Utility Easement, as granted by the Accommodation Agreement and described in Recital M of this Easement Deed. The final location of the Pipeline/Utility Easements shall be determined by those certain City Extraction Approvals, a development agreement (with a term of at least fifteen (15) years) between Mineral Lessee and the City, pursuant to California Government Code Section 65864 *et seq.*, those Other Agency Extraction Approvals, and the development agreement with the City vesting those extraction approvals ("Development Agreement"), with potential modification as set forth herein to accommodate Owners' Surface Development.

b. Relocation of Existing Pipelines. The location of some of the Pipeline/Utility Easements may need to change from time to time as Owners obtain approvals and/or permits from the City and other governmental agencies for Surface Development over time. Owners may, at any time, request that certain existing (as of the Effective Date) utilities and pipelines located within the Property ("Existing Utilities") that conflict with any of Owners' Surface Development Approvals be abandoned and, as appropriate, removed by Owners. These utilities and pipelines may be owned by Mineral Lessee or by other parties such as Pacific Gas and Electric Company ("PG&E") (utilities and pipelines owned by PT&E or other third parties are referred to herein as "Third Party Utilities"); Mineral Lessee may have the right to use Third Party Utilities. Prior to abandonment and removal of any Existing Utilities, Owners must obtain the written consent of the owner of the Existing Utility and, in the instance of Third Party Utilities, of Mineral Lessee. If Mineral Lessee, and, if applicable, the owner of a Third Party Utility, agree to the abandonment and removal, then Mineral Lessee shall not be responsible for the abandonment and removal or any costs of same, and such costs shall be paid by Owners and/or, if applicable, the owner of a Third Party Utility, as agreed between those parties. In the event any of Mineral Lessee's pipelines or utilities need to be relocated and abandoned, such pipeline shall not be abandoned until a replacement pipeline or utility of sufficient size, quality and location has been installed and commissioned (if deemed necessary by Mineral Lessee) to transport gas produced from any location (whether or not from below the Property, the Unit Area or elsewhere) that flows into the pipeline system on the Property.

c. Relocation of Pipelines or Utilities After Completion of Surface Development. At all times after Owners have completed Surface Development of the Property, any pipelines or utility improvements that Mineral Lessee needs to replace, repair or improve (presuming such need was not caused by Owners, in which case Owners shall be entirely responsible for the costs to replace, repair or improve) shall be done at Mineral Lessee's sole cost. Where there are surface improvements in the area of the Pipeline/Utility Easements, including without limitation, landscaping, Mineral Lessee shall be responsible for all incremental costs associated with construction conflicts caused by such improvements and Mineral Lessee shall be responsible for restoring the surface to a condition comparable to that which existed prior to Mineral Lessee's construction activities and for replacing any surface improvements damaged by Mineral Lessee's activities.

4. Environmental Indemnification.

With regard to their respective activities on the Property as permitted by this Easement Deed, the Parties shall comply with all environmental laws, statutes and regulations issued by federal, state and local governmental agencies ("Agencies") and shall conduct their activities on the Property according to environmental standards set by the Agencies, including without limitation, hazardous waste, toxic substances, hydrocarbons and petroleum based substances, water, wetlands, endangered and threatened species and air standards ("Agency Environmental Standards"). The Parties Lessee shall also comply with California Health and Safety Code Section 25359.7. If a Party's use of or activity on the Property results in a loss to any other Party due to a violation of Agency Environmental Standards resulting from use of the Property, whether an enforcement action is brought by an agency or a private citizen, the Party whose use caused the loss shall defend (using legal counsel reasonably satisfactory to the other Party), indemnify and hold harmless the other Party(ies) from the loss and any resulting penalties and fines; and the Party whose use led to the violation shall, at its sole cost, respond to all such violations for its benefit and for the benefit of the other Party(ies), including all clean-up, demolition, detoxification, and disposal and the preparation of any closure or other required plans. In the case of joint or contributory negligence, breach of contract or other fault or strict liability on the part of the Party seeking indemnification, principles of comparative negligence shall be followed and each Party shall bear the proportionate cost of any loss, damage, expense, or liability attributable to such Party's negligence, breach of contract, use or other fault or strict liability. This Section 4 applies only to activities and uses occurring after the Effective Date. There are no third party beneficiaries to this Section 4. The environmental indemnification obligations contained in this Section 4 shall survive the termination of each Parties' interest in the Property and the Pipeline/Utility Easements.

5. Termination of the Pipeline/Utility Easements.

a. Mineral Lessee's interest in the Pipeline/Utility Easements shall terminate when Mineral Lessee releases the Pipeline/Utility Easements to Owners and Mineral Owners. Mineral Lessee shall have the right, in its sole discretion, to elect to abandon any pipeline in place. Upon expiration or termination of its interest in the Pipeline/Utility Easements, Mineral Lessee or its successor, shall promptly and expeditiously execute and acknowledge, on request of Owners, Mineral Owners or their title insurer, a release to Mineral Owners formally releasing of record Mineral Lessee's interests in, and rights under, the Pipeline/Utility Easements.

b. Mineral Owners' interest in the Pipeline/Utility Easements shall terminate at the earlier of (i) when the pipelines are not used to transport hydrocarbons for a period of longer than one hundred eighty (180) days after Mineral Lessee has recorded a release of the Pipeline/Utility Easements, (ii) when the ownership of the Mineral Estate and Surface Estate merge, or (iii) when the Mineral Owners' interest in the Pipeline/Utility Easements is otherwise released.

6. Binding Effect.

The provisions of this Easement Deed shall be binding upon and inure to the benefit of the Parties, their respective heirs, transferees and successors in interest, including, without limitation, all subsequent owners of the Sale Property and the Retained Property, and the burden of the Pipeline/Utilities Easements shall be covenants running with the land of the Property. For purposes of apportioning responsibility and liability for the obligations generally ascribed to "Owner" in this Easement Deed, and unless the context of this Easement Deed otherwise specifically provides, (i) the fee estate owner of the Sale Property shall be responsible and liable only for those obligations, actions and activities arising under this Easement Deed that pertain to or otherwise stem from the ownership of the Sale Property; and (ii) the fee estate owner of the Retained Property shall be responsible and liable only for those obligations, actions and activities arising under this Easement Deed that pertain to or otherwise stem from the ownership of the Retained Property. If the Option Agreement terminates or is terminated prior to RVHHC acquiring any portion of the Property, the term RVHHC shall mean Surface Owners or future developers taking their interest through Surface Owners, and RVHHC shall not have any further rights under this Easement Deed. Mineral Lessee shall have the right to assign this Easement Deed and any of Mineral Lessee's rights hereunder.

7. Mineral Lessee's Right to Authorize Use by Unit Operator; Mineral Lessee and Mineral Owners' Right to Authorize Use by Third Party Utility Providers.

Mineral Lessee shall have the right to authorize the use of the Pipeline/Utility Easements by the Unit Operator for the purposes of conducting Unit operations, so long as the Unit Agreement is in effect. Mineral Lessee and Mineral Owner shall have the right to authorize the use of the Pipeline/Utility Easements by any public or private utility company providing any utilities serving the Well Sites, or as necessary to transport gas to market.

7. Status of Title.

This Easement Deed is made subject to all conditions, covenants, restrictions, leases, easements, licenses, liens, encumbrances and claims of title of record which may affect the Pipeline/Utility Easements.

8. Notices.

All notices, demands, or other communications required or permitted by this Easement Deed or by law to be served on or given to a Party shall be in writing and shall be deemed delivered (i) if personally delivered, upon delivery to the Party to whom directed, (ii) if mailed, upon the expiration of forty-eight (48) hours from the date of mailing in the United

States mail, registered or certified, return receipt requested, addressed to the Party address shown below, or (iii) if faxed, upon receipt of the fax transmittal, transmitted to the Party fax number shown below, provided the machine sending the fax provides a written confirmation of receipt:

If to the Surface Owners, at:	c/o Mr. John Wyro The Wyro Company 40 Valley Drive Orinda, CA 94563 Telephone: 925-254-5246 Fax: 925-254-5299
And to:	Richard and Sue Esperson 398 Crescent Drive Rio Vista, CA 94571
And to:	Mrs. Jean Grimm 35 San Gabriel Dr. Fairfax, CA 94930
With a copy to:	Teri A. Bjorn, Of Counsel Kronick, Moskovitz, Tiedemann & Girard 1675 Chester Avenue, Suite 320 Bakersfield, CA 93301 Telephone: 661-864-3800 Fax: 661-864-3810
And with a copy to:	David G. Kenyon, Esq. 7200 Redwood Blvd., Suite 404 Novato, CA 94945 Telephone: 415-892-1868 Fax: 415-892-1716
If to Mineral Owners, at:	c/o Mr. John Wyro The Wyro Company 40 Valley Drive Orinda, CA 94563 Telephone: 925-254-5246 Fax: 925-254-5299
And to:	Richard and Sue Esperson 398 Crescent Drive Rio Vista, CA 94571
And to:	Mrs. Jean Grimm 35 San Gabriel Dr. Fairfax, CA 94930

With a copy to:	Teri A. Bjorn, of Counsel Kronick, Moskovitz, Tiedemann & Girard 1675 Chester Avenue, Suite 320 Bakersfield, CA 93301 Telephone: 661-864-3800 Fax: 661-864-3810
And with a copy to:	David G. Kenyon, Esq. 7200 Redwood Blvd., Suite 404 Novato, CA 94945 Telephone: 415-892-1868 Fax: 415-892-1716
If to Mineral Lessee, at:	Rosetta Resources Operating, L.P. 1200 17th Street, Suite 770 Denver, CO 80202 Telephone: (720) 946-1315 Fax: (720) 359-9140
With a copy to:	Rosetta Resources, Inc. Attn: General Counsel 717 Texas Avenue, Suite 2800 Houston, TX 77002 Telephone: (713) 335-4017 Fax: (713) 335-4136
And with a copy to:	Stephen R. Finn, Esq. One Market Street, Spear Tower San Francisco, CA 94105 Telephone: 415-442-1251 Fax: 415-442-1001
If to the RVHHC, at:	Rio Vista Hills Holding Company, LLC c/o Lewis Operating Corp. 9216 Kiefer Blvd, Suite 8 Sacramento, CA 95827 Attn: Douglas Mull, Vice Pres. Telephone: 916-363-2617 ext.226 Fax: 916-364-9353
And to:	Lewis Operating Corp. Legal Department 1156 N. Mountain Avenue Upland, CA 91786 Attn: W. Bradford Francke, Esq.

Telephone: 909-946-7538
Fax: 909-946-6725

With a copy to:

Law Offices of Gregory D. Thatch
1730 "I" Street, Suite 220
Sacramento, CA 95814
Attn: Michael Devereaux, Esq.
Telephone: 916-443-6956
Fax: 916-443-4632

9. Choice of Law; Venue.

This Easement Deed shall be governed by and construed in accordance with the substantive and procedural laws of the State of California, excluding any laws that require the application of another jurisdiction's laws. This Easement Deed is entered into and is to be performed in Solano County, California, and accordingly the only appropriate venue for a dispute under this Easement Deed is in the Solano County Superior Court of California. All references to codes in this Easement Deed are to California or City codes, unless otherwise specified.

10. Entire Agreement.

With the exception of the Lease, Unit Agreement, Accommodation Agreement and Declaration of Covenants, Conditions and Restrictions for the Del Rio Hills Project, this Easement Deed (including the Exhibits to this Easement Deed) supersedes any and all other agreements, either oral or in writing, between the parties with respect to the subject matter and contains all of the covenants and agreements between the parties with respect to such matter, and each party to this Easement Deed acknowledges that no representations, inducements, promises or agreements, oral or otherwise, have been made by any party, or anyone acting on behalf of any party, which are not embodied herein, and that no other agreement, statement or promise not contained in, or contemplated by this Easement Deed shall be valid or binding.

11. Modification.

This Easement Deed may be modified or amended only by a written recordable document signed and acknowledged by the (a) then-current Surface Owners, (b) RVHHC or its successor, if any, (c) then-current Mineral Lessee, and (d) then-current Mineral Owners, or by their respective successors-in-interest, transferees or assigns.

12. Partial Invalidity.

In the event a court of law determines that any provision of this Easement Deed, or portion thereof, is prohibited, unlawful, or unenforceable under any applicable law of any jurisdiction, the remainder of the provisions hereof shall remain in full force and effect and shall in no way be affected, impaired or invalidated.

13. Time.

Time is of the essence in the performance of the parties' respective obligations contained in this Easement Deed.

14. Counterparts.

This Easement Deed may be executed in any number of counterparts all of which when executed shall constitute one document.

IN WITNESS WHEREOF, Grantors and Grantees have executed this Easement Deed as of the Effective Date.

GRANTORS;

THE GRIMM-RIO VISTA FAMILY
LIMITED PARTNERSHIP
By_____
General Partner

RICHARD W. ESPERSON also known as Richard W. Esperson, Jr., as Trustee of the Richard W. Esperson and Irene Sue Esperson Family Trust dated October 17, 1991

IRENE S. ESPERSON, as Trustee of the Richard W. Esperson and Irene Sue Esperson Family Trust dated October 17, 1991

MARK ESPERSON

GARY ESPERSON

STEPHEN ESPERSON

KIMBERLY ESPERSON

RIO VISTA HILLS HOLDING COMPANY, LLC
a Delaware limited liability company

By North Mountain Corporation,
a California corporation
Its Sole Manager

By_____
_____

By_____
_____

GRANTEES:

_____
NORMA JEAN GRIMM, also known as Jean Harris Grimm, as Trustee of the Grimm Family Trust dated October 4, 1990 – Survivors Trust

_____
RICHARD W. ESPERSON, JR.

_____
JOAN ESPERSON WEDDELL

_____
DAVID SANTOS

_____
RICHARD SANTOS

_____
STEPHEN ESPERSON

_____

GARY ESPERSON

_____

MARK ESPERSON

_____

SUSAN A. BORGESEN, formerly Susan A. Woodworth

_____

SANDRA J. DICKSON, formerly Sandra Grimm

_____

SHARON E. HARRIS, formerly Sharon Grimm

_____

STEPHEN A. GRIMM, formerly Stephen Grimm

THE GRIMM-RIO VISTA FAMILY LIMITED PARTNERSHIP

By_____
General Partner

_____

JEAN HARRIS GRIMM

_____

DAVID L. SANTOS, as Surviving Trustee of the David L. and Laura E. Santos Revocable Trust dated February 12, 2002

_____

RICHARD W. ESPERSON, also known as Richard W. Esperson, Jr., as Trustee of the Richard W. Esperson and Irene Sue Esperson Family Trust dated October 17, 1991

_____

IRENE S. ESPERSON, as Trustee of the Richard W. Esperson and Irene Sue Esperson Family Trust dated October 17, 1991

ROSETTA RESOURCES OPERATING, L.P., a California limited partnership

By_____

_____

By_____

_____